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PLANT PHYSIOLOGY

VOLUME 27 APRIL, 1952 NUMBER 2

MOLYBDENUM REQUIREMENT OF LEGUMINOUS PLANTS SUPPLIED WITH FIXED NITROGEN

W. R. MEAGHER,[1] C. M. JOHNSON AND P. R. STOUT

(WITH FIVE FIGURES)

Received December 6, 1951

This report offers evidence of the essentiality of molybdenum for the growth of two important members of the Leguminosae. WILSON (**5**) from field experiments, convincingly deduced that the scald disease of beans in New South Wales was due to a straightforward molybdenum deficiency of the bean plant uncomplicated by secondary effects of nitrogen transforming microörganisms as discussed by MULDER (**4**). EVANS *et al.* (**1**) with carefully controlled solution culture experiments demonstrated the essentiality of molybdenum for alfalfa when supplied with sources of fixed nitrogen. HEWITT (**2**) also has reported production of molybdenum deficiency symptoms in lucerne, alsike clover, and red clover.

The particular experiments reported here were undertaken as a necessary adjunct of certain cropping experiments with a low molybdenum supplying soil. Because of the timely reports mentioned above (**1, 2, 5**), the writers first thought that an account of our experiences might be superfluous since the primary intent in conducting these experiments was to determine whether or not molybdenum was essential for the growth of legumes. However, in this work there are several other points of interest regarding the quantitative requirements for molybdenum, the specific symptoms of molybdenum deficiency in beans and peas (which are quite different), and the nature of recovery of injured plants after receiving molybdenum supplements which may be of interest to plant physiologists and perhaps agronomists.

WILSON's suggestion (**5**) that bean seeds imported into low molybdenum areas produced plants which were immune to scald disease because of the higher molybdenum contents of the seed, becomes completely plausible in the light of these experiments. For beans and peas, seeds containing 0.5 microgram molybdenum were found to satisfy completely the physiological requirement for molybdenum during growth of the plant from seed to ma-

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turity. Seeds from commercial sources were found to be highly variable in molybdenum with 3 to 5 micrograms per seed being not uncommon. Molybdenum thus becomes novel from the point of view that we know of no other nutrient element which can be stored in the seed and subsequently be made available to the plant in amounts ranging from sufficient, to a tenfold excess of the mature plant's nutritional needs.

The need for the specific experiments reported here arose from observations on the comparative growth of legumes made by JOHNSON *et al.* (**3**) during studies of a large number of different species of crop plants in pot cultures growing on the same molybdenum deficient soil. The soil had received liberal fertilization with respect to nitrate, phosphate, and potassium. At the time the experiments reported here were started, Johnson *et al.* had grown 30 different kinds of plants. Moderate to very severe injury was obtained for all non-leguminous dicotyledons. By contrast, no evidence of molybdenum stress was shown in any of the legumes included in the series. The legumes were: peas (*Pisum sativum*); beans (*Phaseolus vulgaris*); cowpeas (*Vigna sinensis*); the clovers (*Trifolium repens, Trifolium subterraneum, Melilotus alba*); and trefoil (*Lotus corniculatus*). It was also noted that no evidence of molybdenum stress was shown by any of the monocotyledons included in the series, namely oats (*Avena sativa*), barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), sorghum (*Sorghum vulgare*), and corn (*Zea mays*). Since then, the series has been expanded considerably to cover other species and their growth during different seasons of the year. A report of the comparative molybdenum nutrition of all the species grown on the same soil will be presented elsewhere. However, it should be stated that early generalizations regarding lack of responses to molybdenum by legumes has been consistent in all subsequent experiments; and that among the grasses so far tested only timothy (*Phleum pratense*) has shown response to molybdenum fertilization.

Since the legumes failed to show response to molybdenum fertilization the question arose as to whether they required molybdenum when supplied with fixed nitrogen. At the time, no references in the literature were found wherein a legume had been used in critical demonstrations of molybdenum as an essential element for plant growth. Therefore it appeared necessary to settle the point by experimentation.

In the following experiments, molybdenum deficiency symptoms in peas or beans could not be demonstrated when seed from commercial sources was used. However, by growing a crop on purified culture solutions, and by using the seeds so produced (first generation seed) for further experiments, it was demonstrated conclusively that molybdenum was essential for the growth of beans and peas.

Experimental procedure

Dwarf Horticultural variety beans and Dwarf Telephone peas were grown on purified basal nitrate medium of the following composition in

milliequivalents per liter: K, 7; Mg, 4; Ca, 10; NO_3, 15; H_2PO_4, 2; SO_4, 4. Salts containing the micronutrients were added to give 0.5 p.p.m. B; 0.5 p.p.m. Mn; 0.02 p.p.m. Cu; and 0.05 p.p.m. Zn. Ferrous sulphate to make 0.5 p.p.m. Fe was supplied twice weekly. Macronutrient salt solutions were purified by coprecipitation of molybdenum with copper as sulphides. Pyrex distilled water was used. Comparative growth experiments with tomatoes had shown earlier that less than 0.05 microgram of molybdenum was derived from the salts, containers and all of the water added during growth of the plants.

Seeds were germinated in Pyrex trays with Pyrex supporting rods designed to permit easy removal of the germinating seedlings for transplanting. Four plants were planted in four liters of culture solution in Pyrex beakers having paraffined plaster of Paris covers. First generation seeds

FIG. 1. Bean leaves. Molybdenum deficient (left) and normal (right).

used for most of the experiments contained an average of 0.08 microgram molybdenum per seed for both peas and beans.

With the first generation seed considerable growth was made before signs of molybdenum deficiency appeared. However, after five weeks, abnormalities appeared in the plants having received no molybdenum supplement. Following onset of symptoms each additional day resulted in marked decline of the plants. No symptoms appeared in any of the control plants receiving 0.01 p.p.m. molybdenum (40 micrograms per beaker) nor did symptoms appear in plants grown from commercial seed.

After appearance of molybdenum deficiency symptoms, recovery was effected by supplying molybdenum from Na_2MoO_4 by the following methods: (a) painting a leaflet with a solution 100 p.p.m. with respect to molybdenum; (b) addition of 40 micrograms of molybdenum to the culture solution; (c) immersion of the tip of a leaf in a solution containing 100 p.p.m. molybdenum. Exact procedures for effecting recovery varied between beans

and peas because of different times of onset of deficiency symptoms and because of the different manifestations of the deficiency disease.

Results and discussion

Molybdenum deficiency in beans

Within three days after indications of poor health of bean plants, interveinal chlorotic mottling became pronounced, with the leaf tissue collapsing in the central portions of the interveinal areas. At this time, leaves were fully expanded and of the same size as leaves grown on cultures receiving molybdenum. Figure 1 illustrates the dieback occurring in the molybdenum

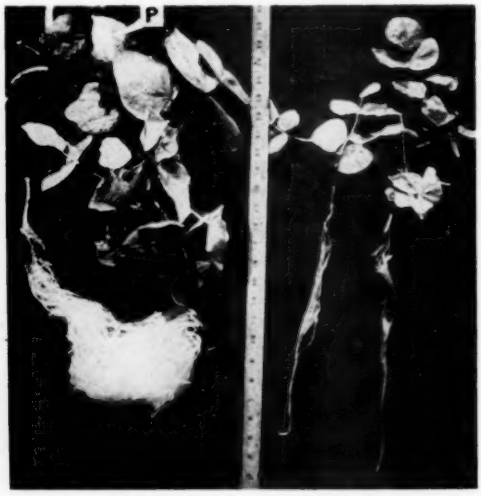

Fig. 2. Removal of molybdenum deficiency in a bean plant by leaf painting. Treated and recovered plant on left. Untreated deficient plants on right. P indicates point of application of Na_2MoO_4 solution.

deficient leaves two weeks later. The normal leaf in figure 1 was taken from a deficient culture which had received molybdenum two weeks before the photograph was taken. There was no noticeable difference between plants grown from commercial seed and plants grown from the special low molybdenum seed in cultures which received 40 micrograms molybdenum. Another point of interest in figure 1 is that green bands remain close to the midrib and veins of the molybdenum deficient leaf even though death has occurred in most of the cells in the interveinal areas. The green bands persisted for several weeks before the entire leaf was dead.

Cultures given 40 micrograms molybdenum three days after deficiency symptoms were first observed showed noticeable greening of the plants on the second day after treatment. Within five days, growth of the recovering

plants compared favorably with those of the control cultures. However, the necrotic areas where cellular collapse had occurred to give a water soaked appearance, did not recover. These particular areas dried out completely but the leaf tissue immediately bordering the dried areas became green, turgid and quite comparable in appearance to normal leaf tissue.

All methods of adding molybdenum gave rise to recovery of the treated plants. Painting or infusion of leaflets gave slower recovery than additions of molybdenum to the culture solutions but with the former treatments general greening of the chlorotic plants took place in about two weeks. Treatments by painting or infusion involved only one plant in a culture and only the treated plant recovered. Other plants in the same culture solution were not affected. The treated leaflets did not seem to recover any earlier than

FIG. 3. Molybdenum deficient and normal bean foliage. See text for significance of root sizes.

the rest of the plant. When greening became evident it was general throughout the plant with the possible exception that the younger leaves were more responsive. Treated plants eventually produced blossoms, pods, and seeds.

Figure 2 (left) shows the result of having painted one half of a single leaflet of a molybdenum deficient bean plant. In order to make the solution stick to the leaf, a thin layer of absorbent cotton was placed on the upper side and moistened with a glass rod dipped repeatedly into the sodium molybdate solution. The same plants shown in figure 2 (right) along with the roots of plants in figure 2 (left) are shown in the culture solution at the left figure 3. Special attention is called to the elaboration of roots by the plant having recovered by reason of leaf painting. There was no influence on the growth of the other plants having roots intermingled in the culture solution. Figure 3 (left) shows the molybdenum deficient plants after removal of the

top of the recovered plant. These deficient plants may be compared with the plants on the right having received 40 micrograms molybdenum at the time of transplanting.

Molybdenum deficiency in peas

As with the beans, commercial pea seed did not produce plants showing evidence of molybdenum deficiency. Also plants from seed produced by previous cropping on molybdenum free culture solutions showed severe molybdenum deficiency—but only after the plants had grown for six weeks. Symptoms of molybdenum deficiency in peas differed considerably from other plants we have observed. Whereas chlorotic mottling is a prominent feature of molybdenum deficiency in all other plant species observed, chlorosis of peas was never obvious until a day or two before the leaf died.

Fig. 4. Normal and molybdenum deficient pea leaves.

Figure 4 shows a normal leaf and three successive stages of injury evidenced by molybdenum deficient peas. The leaf A of figure 4 is from a plant grown on a purified nitrate solution culture supplemented with 0.01 p.p.m. molybdenum. Leaf B shows severe injury with interveinal areas having collapsed; however, the leaves were still green in color. Tendrils appeared alive and healthy but much restricted in growth. Within two to five days after the onset of leaf cell destruction the leaves became dry at the tips as shown by leaf C. Subsequently, drying extended around the leaf margin and stem tissue and tendrils withered as shown by leaf D.

After the first visible evidence of molybdenum deficiency in peas, death of the lower leaves occurred within a week. However, the three or four terminal leaves remained green. Thus the vine continued to grow, forming new terminal leaves but with only the three or four terminal leaves remaining alive.

Figure 5 shows three pea vines which received different treatments with respect to time and methods of addition of molybdenum. Vine A had molybdenum added to the culture when it had attained the height shown at D. After adding molybdenum to the culture new growth proceeded normally as shown at E. Eventually blossoms and pods were formed on similarly recovered pea vines. Vine B of figure 5 shows a plant which never received supplemental molybdenum in its culture solution. Only the uppermost leaves remained alive. Vine C is a plant grown for the same period as vines A and B but its solution received 40 micrograms molybdenum at the time of transplanting.

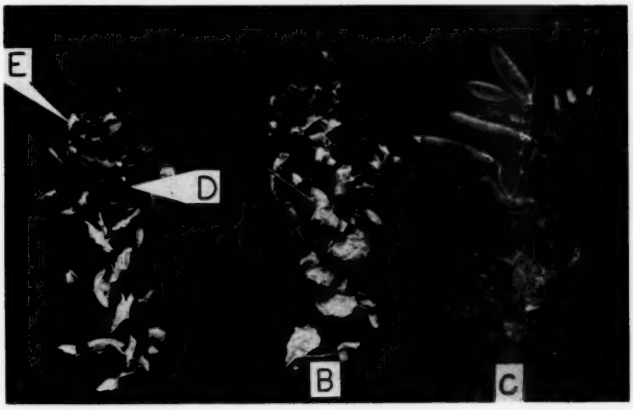

FIG. 5. Pea vines receiving different molybdenum treatments. Vine A, referred to in the text, is on the left.

Summary

Garden peas, *Pisum sativum*, and Dwarf Horticultural beans, *Phaseolus vulgaris*, grown with nitrate as the nitrogen source require molybdenum for the completion of their life cycle.

Because of the large amounts of molybdenum in commercially available seeds (0.5 to 5 micrograms per seed), demonstration of the need of the above species for molybdenum required the use of specially grown low molybdenum seeds. First generation seeds from plants grown on purified culture solutions contained in the order of 0.05 to 0.1 microgram of molybdenum. Severe molybdenum deficiency could be demonstrated in plants grown from such seeds.

During early growth, plants with and without molybdenum showed no visible differences in color or growth rates. Molybdenum deficiency symptoms developed suddenly, becoming definitely noticeable within a single day. Upon addition of molybdenum to culture solutions deficient plants recovered visibly within two days provided the deficiency was manifested only by mottling without necrosis. Painting leaves with a solution contain-

ing molybdenum, or infiltration with a similar solution arrested progressive necrosis, but a period of two weeks was required to show visible signs of improvement. Recovery after painting was general throughout the plant and the specific leaves receiving the treatment appeared to have little special advantage. Bean and pea plants which did not receive molybdenum supplements following visible signs of molybdenum deficiency declined very rapidly with one additional week being sufficient to cause death of most of the interveinal leaf tissue.

Molybdenum is unique among the essential elements in that normal seed of some plants may store, in available form, ten times the total molybdenum needs of the plant to be grown from the seed.

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THE FORMATION OF STARCH IN LEAVES OF *BRYOPHYLLUM CALYCINUM* CULTURED IN DARKNESS

HUBERT BRADFORD VICKERY

(WITH TWO FIGURES)

Received June 28, 1951

In experiments carried out in this laboratory several years ago, it was noted that excised leaves of *Bryophyllum calycinum* collected at daybreak and cultured in water in darkness may increase appreciably in their content of starch (**3**). Such leaves contain a high proportion of organic acids when picked but undergo a marked loss of acidity when kept in darkness for two or three days. The suggestion was advanced that the newly formed starch may have arisen from organic acids as a result of reversal of the glycolytic reactions. Experiments on the normal diurnal variation respectively of organic acids and starch in Bryophyllum leaves (**4, 6**) have lent considerable support to the view that such a course of events is possible, and an unpublished experiment carried out by the late Dr. G. W. Pucher shortly before his death in 1947 showed that starch accumulation in leaves cultured in darkness may at times reach considerable proportions. Accordingly, renewed study has been given to this problem.

Bryophyllum calycinum leaves collected at daybreak were maintained in a closed dark cabinet under a continuous fine spray of water for several days, individual samples being removed for analysis at appropriate times. The leaves, which contained 3.7 gm. per kilo of initial fresh weight of starch at dawn, contained only 1 gm. after a further seven hours in darkness but then began to accumulate starch so that, after 26 hours, they contained 3 gm. and, after 75 hours, 3.6 gm. An even larger relative increase had been observed in the unpublished experiment of Pucher (see also **3**) so that there appears to be no doubt of the capacity of Bryophyllum leaves to behave in this manner, notwithstanding a report to the contrary of VARNER and BURRELL (**5**).

Experimental procedure

Ten samples of leaflets were collected June 29, 1949 by the statistical method (**7**) from plants of the same lot as those described in another paper (**6**) and in the same manner. Collection was begun at 4:00 A.M. (standard time, sunrise at 4:22 A.M.). Zero time was taken as 5:00 A.M. when the control sample was placed in the drying oven, and all samples were in place in the closed sterilized metal cabinet by 5:07 A.M. The samples were held in wire baskets, previously sterilized with steam, supported on two shelves, and water at a constant temperature of 23° C was supplied continuously from two centrally located spray nozzles immediately above each layer of baskets. Sampling times were chosen so as to obtain frequent observations

in the early part of the experiment. As each successive sample was removed, the leaflets were individually blotted as dry as possible, and the sample was weighed and dried at 80° C. The first evidence of rootlet formation at the indentations in the margins of the leaves was seen at 75 hours. At 99 hours, most of the leaflets had developed rootlets some of which were 1 cm. long and, at the end of the experiment at 196 hours, in spite of the fact that these leaflets were relatively young, all save the smallest had rootlets many of which were 3 cm. long.

TABLE I

FUNDAMENTAL DATA ON SAMPLES OF EXCISED *Bryophyllum Calycinum* LEAVES COLLECTED BY THE STATISTICAL METHOD AND SUBJECTED TO CULTURE IN WATER IN DARKNESS.

Culture period	Per sample			Per kilo initial fresh weight				
	Fresh weight at start	Fresh weight at end	Equilibrated dry weight	Equilibrated dry weight	Nitrogen	Organic solids	Ash	Starch
hrs.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
0	128.5	128.5	14.50	112.8	2.83	93.2	11.4	3.74
7	131.8	135.1	14.70	111.5	2.78	91.4	11.1	0.93
14	126.0	132.8	13.65	108.3	2.83	89.1	11.0	1.59
26	129.3	140.0	13.40	103.6	2.81	85.6	11.1	3.05
34	129.6	141.6	13.05	100.7	2.78	83.7	11.0	2.90
50	126.7	137.2	12.50	98.66	2.79	81.8	10.9	3.10
75	127.2	137.7	12.40	97.48	2.75	81.1	10.8	3.63
99	130.7	141.1	12.60	96.40	2.81	79.9	10.9	3.41
147	129.6	143.8	11.90	91.82	2.72	76.1	10.7	3.13
195	131.7	148.6	12.15	92.25	2.79	75.9	10.9	3.20
Mean	129.1				2.79		11.0	
Standard deviation	±2.01				±0.03		±0.20	
Coefficient of variation, %	1.6				1.2		1.8	

The techniques for drying, equilibrating, grinding and storing the samples are described in the previous paper (**6**). They were removed from the air-conditioned room only when subsamples were being weighed for analysis.

Table I gives the fundamental data on the weights and composition of the samples. The accuracy with which they duplicated each other is evidenced by the results for total nitrogen which had a coefficient of variation of 1.2%. Notwithstanding the apparent slight loss of ash constituents to be seen in the observations at zero and at seven hours, the ash weights had the unusually low coefficient of variation of 1.8%. The alkalinity of the ash averaged 256 ± 5 m.eq. per kilo of initial fresh weight (fig. 1), and the data did not reflect the slight loss to be seen in the initial figures for ash. It is possible, therefore, that the lost material consisted merely of dirt on the surface of the leaves which was washed away by the water. The organic solids

show continuous loss owing to the effect of respiration, the data for the first 34 hours (fig. 2) falling upon an approximately straight line; the rate of loss of organic solids then diminished.

The initial behavior of the starch (fig. 2) suggests that, for the first few hours of the experiment, starch was being used in metabolism in a manner

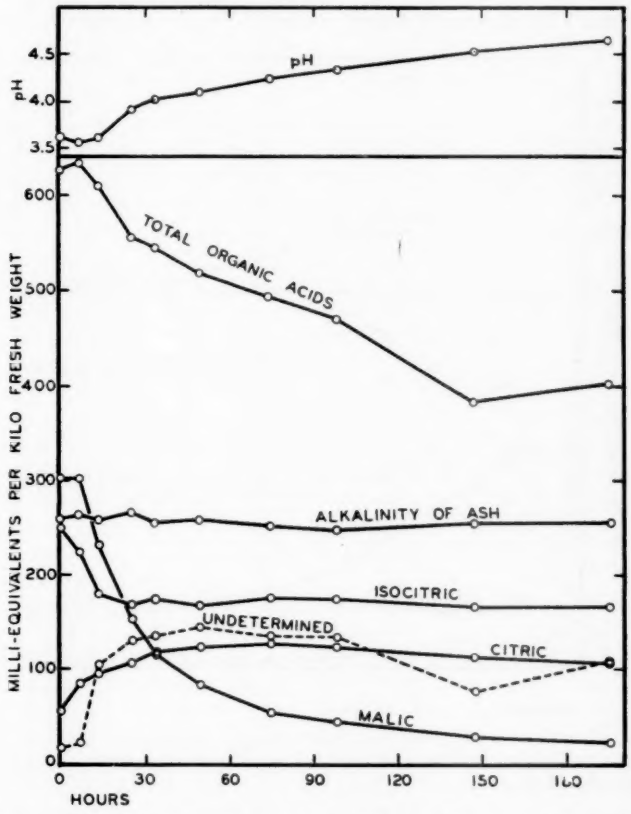

FIG. 1. Changes in the organic acid composition and pH of excised leaves of *Bryophyllum calycinum* cultured in water in darkness. Data are milliequivalents per kilo of initial fresh weight.

that was, in effect, a continuation of its behavior during the night. Leaves closely similar to these had been found to contain approximately 20 gm. of starch per kilo at sunset when exposed to light throughout the day, and it is to be presumed that the 3.7 gm. per kilo found in the present samples at dawn represent the part of this quantity which still remained when the samples were collected.

A significant change in the organic acid composition was initiated shortly after the start of the experiment, for at the end of seven hours citric acid had increased and isocitric acid had decreased (fig. 1). Under the stress set up by the prolonged period of darkness, more of the components of the tissue then became involved in the chemical changes that occurred. Respiration, as evidenced by the loss of organic solids (table I), continued at a high rate, 1 gm. of organic matter (about 1% of the organic solids) being

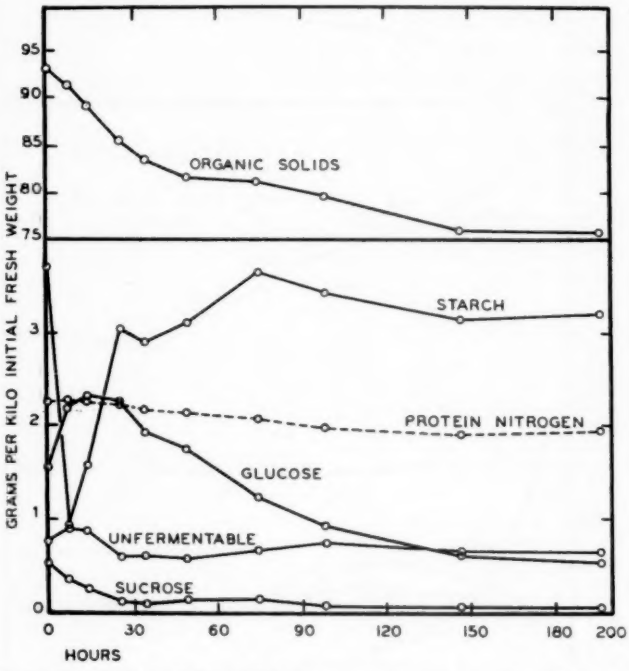

FIG. 2. Changes in soluble carbohydrates, starch, and organic solids of excised leaves of *Bryophyllum calycinum* cultured in water in darkness. Data are grams per kilo of initial fresh weight.

completely oxidized every 3.6 hours for the first 34 hours. Beginning after seven hours, malic acid (fig. 1) diminished rapidly in quantity and there was also a sudden increase in the organic acids of the so-called undetermined fraction. Nevertheless, the total quantity of organic acids present in the system diminished continuously and the pH began to rise. Most striking of all was the formation of an appreciable quantity of starch.

During the second day, the protein (fig. 2) began to be used in the metabolism and, in the interval between 26 and 99 hours, protein nitrogen diminished by about 10% of the quantity initially present; thus about 1.7

gm. of protein disappeared as such during this interval. Subsequently, however, the loss of protein was only slight.

Probably associated with the behavior of the starch is that of the soluble reducing sugar calculated as glucose (fig. 2). This increased by about 0.7 gm. in the first seven hours (the starch diminished by nearly 3 gm.) but then passed through a maximum and subsequently decreased until 99 hours had elapsed. Between 14 and 75 hours, about 1 gm. of glucose disappeared while about 2 gm. of starch were formed. The unfermentable carbohydrate likewise had increased slightly at seven hours and subsequently decreased, but it soon reached a level that remained substantially constant for the rest of the period studied. The quantities involved in the changes of this component were, however, small, being only from 0.2 to 0.3 gm. Sucrose dropped rapidly from its initial level of 0.5 gm. per kilo and only negligible amounts remained after the first day.

The more rapid phase of the general metabolism continued for about 75 hours. By that time the rate of respiration had decreased materially and starch began to diminish slowly. The rate at which malic acid was diminishing also had become slow and citric acid had passed through a flat maximum. Isocitric acid, however, appeared to have reached a new equilibrium level within 26 hours and thereafter changed not at all.

Discussion

To visualize the relative magnitude of the major chemical transformations that occurred, the behavior of malic acid may be considered. Starting at 302 m.eq. per kilo of fresh weight, malic acid diminished to 22 m.eq. after 195 hours. Thus, 280 m.eq. or 18.7 gm. of malic acid per kilo were ultimately converted into other substances. This amount is 20% of the organic solids present in the leaves at the start.

The confirmation of earlier observations on the formation of starch during culture of Bryophyllum leaves in darkness for a prolonged period is of special interest. Synthesis of starch took place contemporaneously with the extensive decrease in malic acid and, because of the obvious close relationship of these two phenomena noted in studies of the normal diurnal variation of acids and starch (**4, 6**), it is natural to associate them in the present case as well. Although the data for starch and for malic acid over the period from 7 hours to 195 hours inclusive are closely correlated mathematically ($r = -0.93$; $r = 0.898$ for $P = 0.001$ with seven degrees of freedom), an inquiry into the relative quantities of starch and of malic acid that underwent change during the successive periods of study suggests that the metabolic relationship is not a simple one. During the first seven hours, starch equivalent to 17 millimoles of glucose (3% of the organic solids) disappeared but there was no change in malic acid. In the next interval of seven hours, starch equivalent to four millimoles of glucose was formed while malic acid dropped by 35 millimoles. During the next 12 hours, starch equivalent to nine millimoles of glucose was formed and 39 millimoles of

malic acid disappeared. The molar relationships in the subsequent intervals are equally irregular. Although the transformation of malic acid into other substances continued at a rate which follows a smooth curve, the changes in starch fluctuated irregularly and by quantities that were in all cases small in comparison with those of the acid. In the absence of studies with tracer elements, no definite conclusion as to the chemical fate of the malic acid can be drawn and none regarding the origin of the starch. The purely analytical method of searching for metabolic relationships breaks down when the changes observed are small in comparison with the total mass of metabolizing material present.

Nevertheless, there are two relationships among the organic acid components of the tissue that merit particular attention. Isocitric acid remains unchanged in quantity during the course of the normal diurnal variation in acidity of Bryophyllum leaves (**4, 6**), but it enters into the system of metabolic changes almost at once as a result of the unusual stresses imposed under the conditions of the present experiment. During the first 26 hours, 82 m.eq. per kilo (5.2 gm.) of isocitric acid or one third of the total quantity present disappeared; this amount is 6% of the organic solids of the tissues. During the remainder of the culture period, the quantity of isocitric acid was constant within the limits of accuracy of the measurement (coefficient of variation 2.3%). The effect is therefore an important one in relation to the total quantity of metabolizing substances in the system and clearly indicates that isocitric acid may under certain circumstances become an active participant in the reactions of the leaf in spite of its apparent inactivity in the course of normal diurnal variation in acidity. The observations suggest that, in the initial phases of culture in darkness for a period beyond the normal length of a night, the position of equilibrium of isocitric acid changes in order to meet the new conditions. This is the first demonstration based on direct analytical determinations that isocitric acid may play an active part in the organic acid metabolism of the Bryophyllum plant.

That the decrease in isocitric acid may be correlated chemically, via an aconitase type of reaction, with the simultaneous increase in citric acid is possible but furnishes at best an incomplete picture of the relationships. The increase in citric acid was only 50 m.eq. (3.25 gm.) in the first 26 hours and, more significantly, continued along a smooth curve until about 70 m.eq. (4.5 gm.) had accumulated after 75 hours. A part, or possibly all, of the newly formed citric acid may have been derived from the relatively much larger quantity of malic acid that disappeared during this period. The possibility that some of the citric acid may have arisen from the carbohydrate components is not excluded.

The other notable organic acid transformation was the formation of a substantial quantity of acids in the undetermined group. Although the data for undetermined acids (fig. 1) are obtained by difference and are subject to the combined analytical errors of the separate determinations, uncompensated error alone could hardly account for more than 10 to 15% of the un-

determined acid apparently formed. It is necessary to assume, therefore, that unknown acid components increased from a level of approximately 20 m.eq. per kilo to about 140 m.eq. at the expiration of 75 hours of culture. This quantity is 8 to 9% of the organic solids if calculated as its equivalent of malic or citric acid. Some of this newly formed acid may have arisen from transformations of malic acid, and some may have been derived from the isocitric acid; other possibilities are also open. Decision on these points must await the identification of the components of this group of reactive substances and also the accumulation of knowledge of the enzyme systems in the leaves.

The more rapid metabolic changes seem to have been approximately completed at the expiration of 75 hours at which time a different sequence of events began. The rate of respiration had dropped, starch began to diminish slowly and both citric acid and the group of undetermined acids passed beyond their flat maxima and began to decrease slowly; malic acid continued to decrease. It is not impossible that these subtle changes in the behavior of the components may be associated with the initiation of the reproductive phase in these leaves, for it was at this point that the first evidence of the formation of rootlets at the indentations of the margins of the leaves was seen. During the remainder of the experimental period, growth of rootlets became increasingly notable. However, it is doubtful that the initiation of this biological event is to be directly attributed to the change in the organic acid composition. Growth of plantlets at the margins of old basal leaves in *Bryophyllum calycinum* is a common observation in plants maintained under greenhouse conditions, and in such leaves normal diurnal variation in acid and starch content is still continuing although probably at a lower intensity than in young upper leaves such as those used in the present study (**2**). Accordingly, it does not seem likely that the unusual organic acid composition of the present leaves at the time the reproductive phase began was, *per se*, the condition which elicited this response.

The present experiment, then, lends further emphasis to the possibilities of rapid and extensive alterations in the organic acid composition of plant leaves in response to artificially applied stimuli. These acids are universally regarded as intermediates in the processes of carbohydrate metabolism, not only in connection with the phenomena of photosynthesis (**1**), but also with those associated with respiration in the more thoroughly investigated animal tissues. Pending the identification of the specific enzyme systems active in Bryophyllum leaves, it is speculative in the extreme to attempt to organize the reactions that are observed into systems analogous to those operative in better known cases. Nevertheless, the behavior of the acids in Bryophyllum leaves is consistent with the view that among the enzymatically controlled reactions are some which bear a close analogy to certain of the changes recognized as components of the well-known Krebs tricarboxylic acid cycle.

The possible substrates of the high respiration observed in these leaves

in the early phase of the culture experiment may be indicated by some of the data. The loss of organic solids in the first 26 hours was 7.6 gm. In this period, glucose increased by 0.7 gm., sucrose decreased by 0.4 gm., and starch at first decreased by 2.8 gm. and then increased by 2.1 gm. It seems unlikely, therefore, that simple sugars or starch played a direct and important role in the respiration at this stage. Malic acid decreased by 150 m.eq. (10.0 gm.) but the total organic acids decreased by only 69 m.eq., a quantity equivalent to only about 4.6 gm. of solids. This may indeed represent respiration loss but there remain 3 gm. of lost organic matter still to be accounted for. The protein nitrogen was substantially unchanged during this period so that significant respiration of protein components did not take place. Therefore nearly one half of the organic matter that underwent complete oxidation during the first day consisted of substances of as yet undetected chemical nature.

Summary

An earlier observation that moderate quantities of starch may be formed in excised leaves of *Bryophyllum calycinum* collected at dawn and cultured for several days in water in darkness has been confirmed. The increase in starch is accompanied by an extensive and continuous loss of malic acid, and by a slow increase of both citric acid and the group of undetermined organic acids to flat maxima. In addition, about one third of the isocitric acid present disappeared within the first 26 hours but, subsequently, no change in the quantity of this substance was observed. There was a net continuous loss of total organic acids.

The loss of organic solids through respiration was rapid for the first 50 hours but subsequently was slow although continuous. The responses of the simple carbohydrates were small in relation to the much larger quantities of organic acids which underwent change. The loss of protein from the tissues was also relatively small.

Because of the complexity of the metabolic changes, it is impossible to conclude that the newly formed starch was derived through chemical transformations from the malic acid which disappeared. Nevertheless this remains a possibility.

Certain changes in the curves after 75 hours of culture, especially that for starch, are tentatively associated with the initiation of rootlet formation at the indentations of the leaf margins.

The observations as a whole indicate the presence in this species of a carbohydrate metabolism which is independent of photosynthesis but which can lead to the synthesis of starch.

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METABOLISM OF INFILTRATED ORGANIC ACIDS BY TOBACCO LEAVES[1]

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The organic acids are key metabolites in plants, for they function in the synthesis of protein, carbohydrate, and fat and serve as photosynthetic and respiratory intermediates. Their metabolism, which has been receiving increased attention, recently has been reviewed by Thimann and Bonner (**12**). The present paper describes the conversion of infiltrated C^{14}-labeled formic, oxalic, glycolic, acetic, malic or citric acid to other acids in tobacco leaves.

Vickery *et al.* (**16, 17**) have excised tobacco leaves, immersed their cut ends in various solutions, and followed the quantitative changes with time in the organic acids of the leaves. The most striking change in the dark was the decrease in malic acid accompanied by an increase in citric acid; all data supported the suggestion that malic acid was the source of the citric acid formed. More recently Vickery and coworkers (**8, 14, 15**) have immersed the cut ends of tobacco leaves in solutions of the potassium salts of malic, citric, succinic, fumaric, pyruvic, lactic, tartaric, malonic, isocitric, oxalic and acetic acids, and have followed the changes in the dark of total organic acid, oxalic, malic, citric and unknown acids, and acetic and isocitric acids when supplied. The changes observed all appeared compatible with the operation of a tricarboxylic acid cycle in the tobacco leaves.

Krotkov and Barker (**6**) allowed a cut tobacco leaf to absorb labeled acetate through the petiole. Much of the acetate disappearing during the 22 hour treatment in the dark was oxidized to carbon dioxide, but part was transformed into an unidentified fraction with the characteristics of an organic acid. Stutz and Burris (**11**) followed the distribution of C^{14} in tobacco leaves supplied with $C^{14}O_2$ in the light and in the dark. In almost all instances, the malic acid had the greatest total and specific activity among the organic acids. Tobacco plants were allowed to fix $C^{14}O_2$ for 15 minutes in the light and then were kept in the dark for four hours in the absence of $C^{14}O_2$. The leaves harvested after 15 minutes in light yielded organic acids with lower C^{14} specific activities than the leaves at the end of the ensuing dark period; evidently high specific activity precursors of the organic acids were formed in the light and transformed to organic acids in the dark.

Materials and methods

Sodium formate labeled with C^{14} was obtained from the Atomic Energy Commission, Isotopes Division, and carboxyl labeled sodium acetate from

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Tracerlab, Inc. The calcium glycolate-2-C^{14} was synthesized by Dr. B. M. Tolbert. Oxalic acid was produced by the controlled heating of labeled sodium formate (**5**). Malic and citric acids were isolated by chromatography on silica gel from the leaves of *Bryophyllum calycinum* which had been exposed to $C^{14}O_2$ in the light.

Tobacco, *Nicotiana tabacum* var. Havana 38, was grown in the greenhouse for one to three months with HOAGLAND and ARNON's nutrient solution number 1 (**3**). Leaves used for infiltration were from 15 to 17 cm. in length and weighed from 3 to 4 gm. Leaves, cut under water, were submerged in a solution of a C^{14}-labeled acid contained in a paraffin-coated dish. The solutions of the calcium or sodium salts of the acids were adjusted to pH 5.5 with H_2SO_4, and one ml. of the solutions gave 5,000 to 20,000 counts per minute when counted with a windowless flowing gas counter. The dish containing the leaf was placed in a vacuum desiccator, evacuated to about 15 mm. Hg residual pressure, allowed to stand for 30 to 60 seconds, and then the vacuum was released slowly. Successful infiltration was achieved only when the leaf blade was uniformly translucent but the main rib and veins showed no indication of infiltration; the leaf then transpired the infiltrated water rapidly but did not wilt when the petiole was immersed in water. Leaves from individual plants were tested to determine the optimum pressure for infiltration. The infiltrated leaves were rinsed twice with distilled water, blotted with paper towels, and placed in a hood. They were uncovered for illumination or were placed in a ventilated cardboard drum for darkening. The leaves transpired their excess water in one to four hours, and they were allowed to metabolize for 30 minutes after they regained their initial appearance. The leaves were then frozen with liquid air or solid CO_2, ground, and extracted for organic acid analysis.

A slurry of the frozen, ground tissue was made in 0.5 *N* H_2SO_4 and 7 *N* H_2SO_4 was added to pH 1, a pH low enough to liberate the free acids. The slurry was mixed with an equal weight of iron-free (HCl washed) celite 545, and 1 : 1 butanol : chloroform, equilibrated with 0.5 *N* H_2SO_4, was added and mixed (100 ml. total solvent per gm. original wet leaf was used for the slurry and for washing). This slurry was placed on a column, and the cylinder of celite was washed with the additional acid-equilibrated butanol-chloroform when the original solvent from the slurry had passed through it. The effluent was caught in 0.1 *N* NaOH (10 ml. per 4 gm. original tissue) to prevent esterification. The acids were titrated, the salts were extracted into water, the solution was concentrated, and the free acids were liberated and mixed with silica gel essentially as described earlier (**4, 11**). In this second purification the free acids were eluted from the silica gel with 1 : 1 tertiary amyl alcohol : chloroform equilibrated with 1 M Na_2SO_4; the total number of ml. of the solvent collected did not exceed the weight in grams of original leaf tissue used.

For the separation of the organic acids a column containing 1 gm. of silica gel was prepared as described earlier (**4, 11**) with about 1.5 gm. of

0.5 N H_2SO_4 (stationary solvent) added to each gm. of silica gel. Mallinckrodt's silicic acid for chromatography can be employed, but the amounts of silica gel and aqueous phase used will differ from those described here (**7**). The acids in the tertiary amyl alcohol : chloroform solvent were added to the column and fractions of the eluting solvent, 35% n-butanol in chloroform mixture equilibrated with 0.5 N H_2SO_4, were collected with an automatic fraction collector. Water, free of carbon dioxide, was added to each tube, and the contents were titrated with 0.05 N $Ba(OH)_2$. Succinic and acetic acids, which are eluted quickly from the chromatographic columns, may be contaminated with other acids, so the tables designate them as the succinic fraction and the acetic fraction.

Samples from the experiments with labeled malic and citric acids were converted to $BaCO_3$ which was distributed on aluminum planchets for counting (**11**). In all other experiments, aliquots of the titrated acids were dried directly on the aluminum planchets. Specific activities were calculated from the amount of carbon present in the individual acids as indicated by their titration. All samples were counted with a windowless flowing gas counter: the counting errors were less than 10%.

Results

A tobacco leaf was infiltrated with C^{14}-labeled formic acid and then was exposed to light for 40 minutes. From the analyses of the organic acids recovered (table I), it is apparent that during the 40 minute period the carbon of formic acid was distributed into a number of other acids. The assimilation of the C^{14} into malic acid was particularly striking, for at the end of the experiment there was far more C^{14} in malic acid than in the formic acid remaining. Not only the total activity but also the specific activity of malic acid was very high. The citric acid in this leaf was abundant, but picked up much less of the C^{14} than did the malic acid. Oxalic acid, glycolic acid, and the succinic fraction each had a higher specific activity than the citric acid. Little glycolic acid is present in tobacco leaves, but it had a high specific activity. Although it may have been formed by the reversal of glycolic and glyoxylic acid dehydrogenases, for the combined activity of these enzymes yields formic acid and carbon dioxide from glycolic acid, the data do not establish that this was the pathway of glycolic acid formation. Degradation of glycolic acid to determine the position of the label derived from formic acid might indicate the pathway of formation of the compound; it is known (**10**) that fixation of $C^{14}O_2$ rapidly yields glycolic acid uniformly labeled.

Formic acid obviously is a very active metabolite in tobacco leaves, for most of it disappeared in 40 minutes and much of its radioactivity appeared in malic acid. Whether this conversion to malic acid involved an initial oxidation to carbon dioxide and a photosynthetic fixation of the CO_2, or whether formic acid itself reacted in malic acid formation cannot be answered by the data, but it seems very likely that a direct conversion without

TABLE I

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM TOBACCO LEAVES INFILTRATED WITH C^{14}-LABELED FORMIC ACID AND EXPOSED FOR 40 MINUTES IN THE LIGHT.

Organic acid	Acid	Total counts/min.	Counts / min. × mg. C
	mg.		
Formic	0.53	1,530	11,100
Succinic fraction	0.85	520	1,510
Oxalic	4.16	920	830
Glycolic	0.83	690	2,620
Malic	10.80	25,400	6,570
Citric	14.75	2,360	428

formation of CO_2 was involved. The formic acid remaining had a lower specific activity than that infiltrated, so apparently formic acid is a normal metabolite in the tobacco leaf.

Table II gives the distribution of C^{14} after infiltration of C^{14}-labeled oxalic acid into a tobacco leaf. Oxalate is far from an active metabolite, for after 2.5 hours exposure to oxalate in the dark only a very small percentage of the radioactivity had moved from oxalic acid into the other organic acids. The oxalic acid had a specific activity at the end of the experiment about 40 times as high as that of malic and citric acids, and the succinic fraction acquired virtually no C^{14}. The conclusion of VICKERY *et al.* (**15, 16**) that oxalic acid is an end product and a rather inert compound in the tobacco plant is well verified by these tests with the labeled acid.

Pucher *et al.* (**9**) demonstrated that the concentration of oxalic acid increased rapidly in the buckwheat plant in the early hours of the day. It might be implied that oxalic acid is an active intermediate in the buckwheat plant, although the data also are compatible with the concept that the oxalic acid accumulates as a metabolic end product. Table III shows the distribution of C^{14} among the organic acids of buckwheat leaves infiltrated with

TABLE II

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM TOBACCO LEAVES INFILTRATED WITH C^{14}-LABELED OXALIC ACID AND EXPOSED FOR 150 MINUTES IN THE DARK.

Organic acid	Acid	Total counts/min.	Counts / min. × mg. C
	mg.		
Succinic fraction	10.30	23	5
Oxalic	4.86	12,000	9,300
Malic	3.94	314	225
Citric	1.64	163	266

TABLE III

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM BUCKWHEAT LEAVES INFILTRATED WITH C^{14}-LABELED OXALIC ACID AND EXPOSED FOR 40 MINUTES IN THE LIGHT OR 180 MINUTES IN THE DARK.

Organic acid	Acid		Total counts/min		Counts min. × mg. C	
	40 min.	180 min.	40 min.	180 min.	40 min.	180 min.
	mg.	*mg.*				
Volatile acids	3.34	26.20	64	72	47	8
Succinic fraction	0.12	0.62	95	960	1,900	3,830
Oxalic	1.80	15.50	5,630	160,000	11,750	38,800
Glycolic	0.10	0.66	53	1,470	1,700	7,000
Malic	0.92	6.36	377	1,920	1,130	840
Citric	0.31	2.12	60	168	520	210

C^{14}-labeled oxalic acid and kept in the light for 40 minutes or in the dark for three hours. Although appreciable C^{14} passed into succinic, glycolic and malic acids, it is apparent that the total C^{14} and the C^{14} specific activity remained much higher in the oxalic acid than in any other acid. The conversion of oxalic to other acids is several times more rapid than in tobacco and may account for substantial turnover of carbon in buckwheat. The conversion to glycolic acid is noteworthy, for glycolic acid is an active metabolite in leaves and much of the carbon interchanged from oxalic to other acids may pass through it.

Glycolic acid-2-C^{14} was infiltrated into tobacco leaves and these were exposed for 60 minutes in the light (table IV). After 60 minutes the glycolic acid was nearly exhausted, but its specific activity remained higher than the other acids recovered. The total C^{14} and C^{14} specific activity were much higher in malic acid than in citric acid. Oxalic acid had a high specific activity.

When glycolic acid was infiltrated and the leaf was kept in the dark for 180 minutes, the quantitative distribution of C^{14} among the organic acids

TABLE IV

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM TOBACCO LEAVES INFILTRATED WITH C^{14}-LABELED GLYCOLIC ACID AND EXPOSED FOR 60 MINUTES IN THE LIGHT.

Organic acid	Acid	Total counts/min.	Counts min. × mg. C
	mg.		
Acetic fraction	0.78	36	115
Succinic fraction	0.24	42	430
Oxalic	0.89	128	540
Glycolic	0.09	33	1,160
Malic	1.28	215	470
Citric	0.35	16	122

TABLE V

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM TOBACCO LEAVES INFILTRATED WITH C^{14}-LABELED GLYCOLIC ACID AND EXPOSED FOR 180 MINUTES IN THE DARK.

Organic acid	Acid	Total counts/min.	Counts min. × mg. C
	mg.		
Acetic fraction	0.93	167	450
Succinic fraction	0.40	133	820
Oxalic	1.31	176	500
Glycolic	0.10	42	1,330
Malic	3.99	493	350
Citric	0.43	230	1,420

was distinctly different (table V) from that observed in the light. In the dark as in the light the acetic and succinic acid fractions became radioactive. Oxalic acid was abundant and actually had a specific activity higher than malic acid—it is evident that the conversion of glycolic to oxalic acid occurs both in light and in dark. The most striking result appears in the relative specific activities of malic and citric acids. Whereas in the light, malic acid had a specific activity almost four times higher than citric acid, in the dark citric acid had a specific activity four times higher than malic acid, although it had lower total C^{14}.

In their detailed examination of the metabolism of C^{14}-labeled glycolic acid by Scenedesmus, SCHOU *et al.* (**10**) observed that both malic and citric acids accumulated less than half as much C^{14} in the dark as in the light; in each instance there was about half as much C^{14} in citric as in malic acid. The observations with tobacco are compatible with the suggestion of BENSON and CALVIN (**1**) and CALVIN (**2**) that a tricarboxylic acid cycle, active in the dark, may be suppressed by light. The assumption must be made that glycolic acid, in addition to its oxidation by glycolic and glyoxylic acid dehydrogenases, can participate in a tricarboxylic acid cycle. This assumption is supported by the observation of SCHOU *et al.* (**10**) that over twice as much C^{14} from glycolic acid accumulated in glutamic acid in the dark as in

TABLE VI

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM TOBACCO LEAVES INFILTRATED WITH C^{14}-LABELED ACETIC ACID AND EXPOSED FOR 210 MINUTES IN THE DARK.

Organic acid	Acid	Total counts/min.	Counts min. × mg. C
	mg.		
Acetic	0.84	300	895
Oxalic	0.67	4	18
Malic	4.12	830	560
Citric	1.90	690	970

TABLE VII

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM TOBACCO LEAVES INFILTRATED WITH C^{14}-LABELED MALIC ACID AND EXPOSED FOR FOUR HOURS IN THE DARK.

Organic acid	Acid	Total counts/min.	Counts min. × mg. C
	mg.		
Succinic fraction	0.48	280	1,440
Malic	1.12	4,170	10,400
Citric	1.13	957	2,260

the light, a fact indicative of high activity of a tricarboxylic acid cycle in the dark and its partial suppression in the light.

When carboxyl labeled acetic acid was infiltrated and the leaf was kept in the dark for 210 minutes (table VI), citric acid had almost twice the specific activity of malic acid, a result much like that observed when glycolic acid was metabolized in the dark. These two-carbon acids may be used in similar fashion in the formation of malic and citric acids. However, as glycolic acid transfers considerable C^{14} to oxalic acid and acetic acid transfers little, it may be assumed that acetic acid yields little glycolic acid. Any substantial reduction of glycolic to acetic acid thus appears highly unlikely. The higher specific activity of citric acid compared with acetic acid indicates that acetic acid is a normal metabolite of tobacco leaves, for only newly produced acetic acid could dilute the radioactivity of the acetic acid infiltrated to a specific activity lower than that of citric acid. In the experiments of KROTKOV and BARKER (**6**) the dilution of the acetate was insufficient to establish this point.

PUCHER, VICKERY *et al.* (**8, 16**) have presented much evidence indicating that, in the dark, malic acid is converted to citric acid in tobacco leaves. The isotopic technique offers an independent means of checking this observation. Malic acid labeled with C^{14} was infiltrated into a tobacco leaf and the leaf was kept in the dark for four hours. Table VII indicates the spe-

TABLE VIII

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM TOBACCO LEAVES INFILTRATED WITH C^{14}-LABELED CITRIC ACID AND EXPOSED FOR FOUR HOURS IN THE DARK.

Organic acid	Acid	Total counts/min.	Counts min. × mg. C
	mg.		
Succinic fraction	1.99	63.2	78
Oxalic	0.45	5.6	47
Glycolic	0.24	0.4	6
Malic	4.25	15.5	10
Citric	2.36	1,645.0	1,850

cific and total activities of the acids. As malic acid was infiltrated it had the highest specific activity, but citric acid also had a high specific activity. These data leave no question that carbon from malic acid can be used in the synthesis of citric acid in the darkened tobacco leaf. The small amount of succinic acid formed could have arisen by direct reduction of malic acid, but the higher specific activity in citric acid compared with succinic acid is compatible with the alternative that the succinic acid was formed by way of the tricarboxylic acid cycle.

The specific activities of acids isolated from leaves infiltrated with C^{14}-labeled citric acid and kept four hours in the dark are given in table VIII. The citric acid was converted to other acids sluggishly when compared with acids such as formic, acetic and malic. After four hours the citric acid had 24 times as high a specific activity as the next most active fraction. Malic acid was formed slowly from the citric acid. Less activity appeared in glycolic than in oxalic acid in four hours.

Discussion

The data presented illustrate clearly the rapid interconversion of the organic acids in the tobacco leaf. However, there are certain limitations to the experiments that should be considered. First, the tests have been made at pH 5.5 only, and penetration of the labeled compounds into the cells may be better at a lower pH. Second, although incorporation of C^{14} via CO_2, arising from oxidation of the labeled acids, is probably of minor importance, such incorporation is not distinguished from incorporation directly of intact carbon chains. Third, compounds other than the organic acids separated unquestionably assimilate a substantial fraction of the C^{14} from the infiltrated acid. Fourth, lack of replication, high sampling error and variation in time of exposure preclude assertion that the values reported are absolute, although the data are adequate to show the general magnitude of the interconversions of the organic acids in detached tobacco leaves.

With the above limitations defined, an examination of the data indicates a number of clear-cut interconversions of organic acids by the tobacco leaf. One of the most striking is the rapid conversion of formic to malic acid. The data in addition to revealing the rapid utilization of formic acid indicate that it is a normal metabolite in the tobacco leaf. It may arise from the oxidation of glycolic and glyoxylic acids (**13**).

The sluggish response of the leaves to infiltrated oxalic acid is not surprising, for most previous work has indicated it to be an end product of metabolism. The problem of permeability may be a particularly pertinent one here, for the pK_a of oxalic acid is well below that of the other acids used. Assuming that oxalic acid penetrated effectively, there is nothing to indicate it is an active compound in tobacco; however, in buckwheat it may be an important intermediate in acid metabolism.

Malic acid, citric acid and acetic acid (as an active derivative) participate in the tricarboxylic acid cycle. Much evidence suggests the existence

of a tricarboxylic acid respiratory cycle in plant tissues, although detailed support for its function in any single plant tissue has not been marshalled. Determination of the fate of infiltrated labeled acids may reveal the functioning of a cyclic respiratory mechanism. Acetic acid quickly transferred C^{14} to malic and citric acids. If the citric acid arose by condensation of active acetate with oxalacetic acid and then gave rise to malic acid through the usual tricarboxylic acid cycle, both the malic and citric acid would be labeled with C^{14}. The high specific activity of the citric acid fits the concept well that it was formed from active acetate by the condensing enzyme, and the lower radioactivity of malic acid is compatible with its production from the citric acid.

When labeled malic acid was infiltrated into tobacco leaves kept in the dark, citric acid acquired C^{14}, a result one would predict if a tricarboxylic acid cycle were operative. The fact that the succinic acid fraction recovered had a lower specific activity than the citric acid is compatible with its formation from citric acid in the cyclic oxidation process, though it does not prove that it did not arise from reduction of malic via fumaric acid. When the results from the infiltration of labeled citric acid are compared with those from the infiltration of malic acid, it is apparent that in four hours in the dark the succinic fraction had a relatively higher specific activity when derived from malic than from citric acid. Although caution must be used in interpreting the data in absolute terms, these results raise some questions regarding the activity of the cycle. The very low C^{14} content in malic acid four hours after labeled citric acid was infiltrated is indicative of a slow operation of a tricarboxylic acid cycle at best, although permeability difficulties may be evoked in explanation. In short, the data are compatible with the operation of a tricarboxylic acid cycle in detached tobacco leaves but do not indicate that it functions rapidly; its part in the overall respiration of the plant remains to be established.

Summary

Detached leaves of tobacco were vacuum infiltrated with solutions of C^{14}-labeled formic, oxalic, glycolic, acetic, malic or citric acids adjusted to pH 5.5. After the leaves metabolized in the light or dark, their organic acids were recovered and were analyzed for C^{14}.

Formic acid apparently is a normal metabolite in tobacco leaves. It was converted rapidly to other organic acids and particularly high levels of C^{14} appeared in malic acid.

Oxalic acid was rather inert in tobacco but was more active in buckwheat leaves.

When labeled glycolic acid was metabolized in the dark, the ratio of C^{14} in citric/malic acid was markedly higher than in the light. Both in light and dark much C^{14} from glycolic acid accumulated in oxalic acid.

In the dark, acetic acid yielded citric acid of about twice the specific activity of malic acid. Acetic acid did not appear to be oxidized by way of glycolic acid.

Malic acid was converted to citric acid by the darkened tobacco leaf.

Citric acid was converted sluggishly to other organic acids.

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ELECTRICAL AND CURVATURE RESPONSES OF THE AVENA COLEOPTILE TO UNILATERAL ILLUMINATION[1]

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(WITH SIX FIGURES)

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Introduction

One fact that is agreed upon in the literature regarding the mechanism of phototropism is that the growth curvature results from an unequal distribution of auxin. There are at least three hypotheses as to how unilateral illumination causes the unequal distribution: 1. Photoinactivation of auxin on the lighted side of the plant. 2. A differential change in the rate of synthesis of auxin on the lighted and shaded sides. 3. Unilateral displacement of auxin toward one of the sides. It is the last of these mechanisms with which this paper will be primarily concerned.

If lateral transport of auxin serves as a mechanism for phototropism, it requires a transverse polarity which is induced by the tropic stimulation. This is essentially a statement of the familiar Cholodny-Went theory, which applies to other tropisms as well as phototropism. One logical possibility is that such a transverse polarity is electrical in nature. Because of this possibility, the transverse electrical changes which occur in the Avena coleoptile as a result of most of the tropic stimuli have been established. The results of these studies have been reviewed recently by Schrank (**10**).

Apparently only two investigators, (Oppenoorth, **6** Schrank **8**), have attempted to measure the transverse electrical changes resulting from stimulation of the Avena coleoptile by unilateral illumination. Oppenoorth's findings (**6**) indicate that the lighted side of the Avena coleoptile becomes electropositive to the shaded side when the plant is unilaterally illuminated with a dosage of light which causes positive phototropic curvature (500 mcs). Schrank (**8**), on the other hand, measured the changes in the transverse electrical polarity in the apex of the Avena coleoptile resulting from continuous unilateral illumination and found that the lighted side became electronegative to the shaded side.

It is the purpose of this investigation to make additional measurements of the electrical changes of the Avena coleoptile when it is stimulated by a dosage of light which causes positive phototropic bending and to observe the electrical changes resulting from illumination which causes negative bending. If the transverse electrical polarity indeed serves as a polar force which directs the auxin to the side of the coleoptile which becomes convex, the orientation of the light induced polarities in these two instances should be opposite.

[1] Aided by a grant from the University of Texas Research Institute.

Materials and methods

Plants of *Avena sativa*, Victory strain (U. S. Department of Agriculture, C.I. 2020, kindly supplied by the U. S. Department of Agriculture) were grown in the usual manner, (**7**). Intact seedlings with straight coleoptiles 30 millimeters (± 1 mm.) long and which had a well developed root system were used. All experiments were performed in a dark room which was illuminated by a ruby glass neon tube which emits only wave lengths longer than 6074 Å. The temperature was kept at 24° C. (± 3° C) throughout the investigation with variations of not more than 0.5° during any one experiment.

The apparatus used has been described previously (**7**). Two types of contacts were used: 1. Short tapered glass tubes were attached to electrode cups, which were filled with Shive's solution, so that the electrical connection between tube and tissue was a meniscus of this solution. The open ends of these tubes were one millimeter in diameter. Two of these contacts were used in each experiment. They were placed on opposite sides of the coleoptile and were frequently moved laterally to keep the bending plant properly spaced between them. The contact between the light source and the coleoptile was moved during illumination. 2. The second type of contact was a combination holder for the plant and contact cup. This combination was filled with Shive's solution to the desired level for making electrical contact. Only one of these contacts was used for each experiment and it was placed basally to the other two contacts. The electrodes were isoelectric (± 1 mv.) zinc–zinc sulphate half cells. Electrical measurements were made with a DUBRIDGE vacuum tube voltmeter (**2**).

In all experiments the coleoptiles were stimulated by unilateral illumination after a 30 minute control period during which electrical and curvature measurements were made on the unstimulated plant. A 10 watt frosted G. E. mazda lamp and a similar 100 watt lamp were placed at distances from the seedling such that when they were activated for two minutes the plants received 200 and 31,200 mcs respectively. A piece of black paper was kept on the shaded side of the plant during illumination. Each experiment was 90 minutes long. Electrical measurements and tip movements were recorded every five minutes. Tip movements were measured by means of a horizontal ocular micrometer mounted in a horizontal microscope and the curvature was checked at the end of each experiment by the JUDKINS method (**4**).

Results

ELECTRICAL POLARITY CHANGES WHEN THE PHOTOTROPIC RESPONSE IS POSITIVE

In this series of experiments, consisting of three groups, the coleoptiles were stimulated by 200 mcs of unilateral illumination. According to DU BUY and NUERNBERGK (**3**) this dosage of light should cause positive phototropism.

GROUP I.—In the first group, the basal contact was placed 10 millimeters below the apex. This reference contact was connected to ground. The two apical contacts were always placed directly opposite each other on the

lighted and shaded side of the coleoptile about one millimeter below the apex. The apical contacts were alternately connected to the control grid of the duBridge voltmeter. In this way the longitudinal voltages between the apical and basal contacts were measured on both the lighted and shaded side of the coleoptile.

Thirteen experiments were completed in this group. Seven coleoptiles showed positive phototropic bending. The curves in figure 1 A show the typical changes in the longitudinal electrical polarities of the seven. Curve

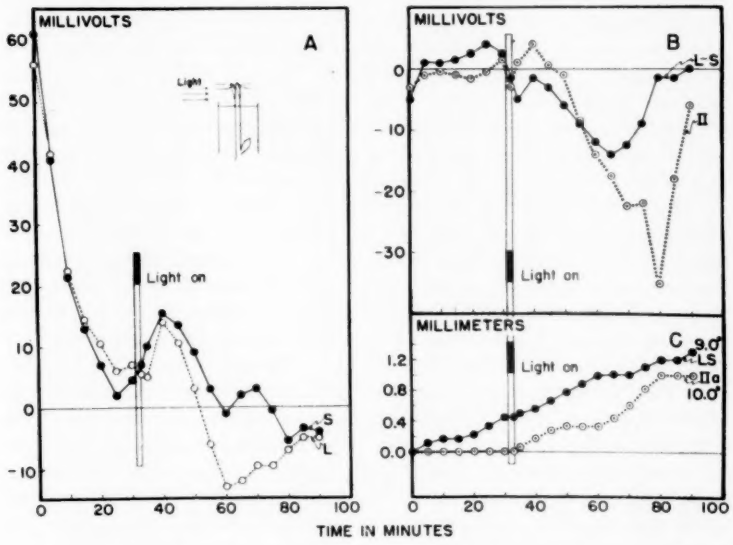

FIG. 1. Electrical and curvature responses to stimulation by 200 mcs of unilateral illumination with contact positions shown by inset. Figure 1 A shows changes in the longitudinal polarity. Curve L on the lighted side and curve S on the shaded side. Figure 1 B shows the transverse electrical polarities. Curve L–S was obtained by subtracting curve S from curve L in figure 1 A. Curve II was similarly obtained from another experiment. Figure 1 C shows curvature responses in the two experiments whose electrical responses are shown in figure 1 B.

L represents the longitudinal polarity of the lighted side, and curve S the corresponding polarity of the shaded side. The orientation of this polarity prior to stimulation is the same as that found by WILKS (**13**). The immediate effect of light stimulation shown by curves L and S is a slight increase in the longitudinal potential. This effect was not typically observed and is probably a manifestation of individual variation of the plant involved. This is followed by a gradual decrease in the polarity, which lasts for about 20 minutes and results in a reversal of polarity. Toward the end of the experiment an increase is again noted especially in curve L.

Curves showing the differences in the longitudinal polarities between the

lighted and shaded side are shown in figure 1 B. (These curves were always obtained by subtracting the longitudinal polarities of the shaded side from those of the lighted side and will hereafter be referred to as transverse polarities.) Curve L–S was obtained from curves L and S in figure 1 A, and curve II was similarly derived from another experiment in this group for which the individual longitudinal polarities were not plotted. Both of these curves stay very nearly at zero during the control period. This means that the magnitudes of the polarities on the lighted and shaded side remain essentially the same. Immediately after stimulation there is a tendency for the lighted side to show a transitory period of positivity to the shaded side. After this the transverse polarity gradually increases with the lighted side electronegative to the shaded side. Toward the end of the experiment the polarity approaches zero again. From the data in figures 1 A and 1 B it appears evident that unilateral illumination affects the longitudinal electrical polarity on opposite sides of the Avena coleoptile similarly, but the magnitude of the change on the lighted side is greater than it is on the shaded side.

The curvatures of the coleoptiles whose electrical responses have just been described are shown in figure 1 C. These curves are labelled to correspond to the electrical curves. Both of the curves show positive bending. The horizontal tip displacement of curve LS is more than for curve IIa, while the magnitudes of the angular curvatures (shown at the end of the curves as degrees) are reversed. This is explained by differences in the distance of the zone of bending from the apex. Caution must always be exercised when interpreting curvature measured by the horizontal displacement of the tip because a slight bending in the basal region can completely mask a larger angular bending in the apex. The bending indicated by the curve LS prior to stimulation is due to slight basal curvature. For this reason there is no way of detecting from curve LS when phototropic bending in the apical region starts. Fortunately, this complication appears in only a few instances.

Group II.—In this group of experiments the reference contact was placed at the base of the coleoptile and connected to ground. The more apical contacts were placed directly opposite each other 10 millimeters below the apex (inset in fig. 2). These contacts were connected to the control grid of the duBridge voltmeter. Seven out of a total of nine coleoptiles showed positive phototropic bending. Results of a typical experiment are shown in figure 2. Changes in the longitudinal polarities are represented by curves L and S in figure 2 A. In all instances the longitudinal polarity was normally oriented at the beginning of the experiment (**13**). As indicated in figure 2 A most of the data show a rapid decrease in polarity immediately after stimulation which is followed by a gradual increase. This is true for both the lighted and shaded sides.

The corresponding L–S curve is shown in figure 2 B. During the control period (before stimulation) the transverse electrical polarity remains close to zero. Immediately after stimulation the lighted side becomes electronegative to the shaded side. This change lasts for only a few minutes and

does not appear in any of the other experiments in this group. The lighted side then becomes electropositive to the shaded side and remains so for about 30 minutes. This change was characteristically observed. However, before the experiment is completed the transverse polarity returns to zero.

The curvature response in this experiment is presented by curve LS in figure 2 C. Slight movements of the tip occurred before stimulation. Such small movements are attributed to circumnutation. It should be noted that the movement of the tip in the phototropic response does not follow a smooth course. This often occurs in tropic bending. The transverse electrical po-

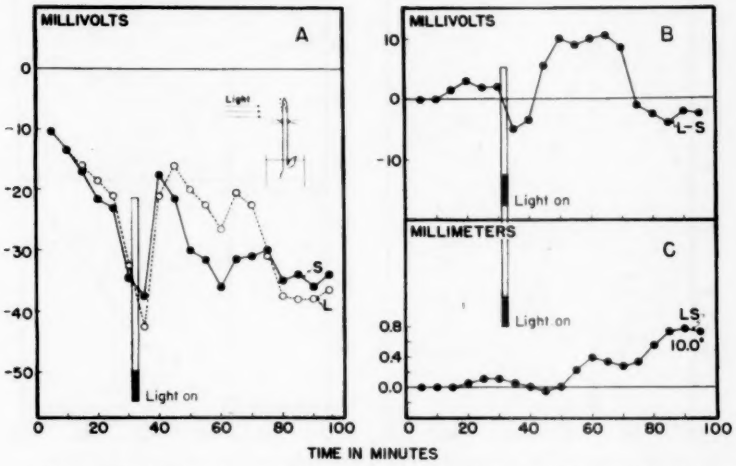

FIG. 2. Electrical and curvature responses to stimulation by 200 mcs of unilateral illumination with contact positions shown by inset. Figure 2 A shows changes in the longitudinal polarity. Curve L on the lighted side and curve S on the shaded side. Figure 2 B shows the transverse electrical polarity which was obtained by subtracting curve S from curve L in figure 2 A. Figure 2 C shows the curvature responses in the same experiment.

larity changes in this series seem to precede the curvature responses, though it was not possible to detect the exact time that curvature started in all instances. The reasons for this have been presented.

GROUP III.—In this group the reference contact was placed at the base of the coleoptile, and the apical contacts were placed about 1 millimeter below the apex. This group consists of six experiments. All showed positive phototropic bending, and in every case the lighted side became electronegative to the shaded side. Results of a typical experiment are shown in figure 3.

Changes in the longitudinal polarity are given as curves L and S in figure 3 A. The polarity was typically oriented at the beginning of the experiment in this case (apex negative to base). The first effect of unilateral stimulation was to increase the longitudinal electrical polarity of both sides

(Increase in polarity as referred to its natural orientation). After a time, however, the voltage returns to its orginal magnitude. Curve L–S in figure 3 B shows the changes which occur in the transverse polarity. The immediate effect of stimulation is to cause the lighted side to become electronegative to the shaded side. After 25 minutes the transverse voltage returns to normal.

The curvature responses of the same coleoptile are shown in figure 3 C by curve LS. The rate of tip movement is more uniform in this case than

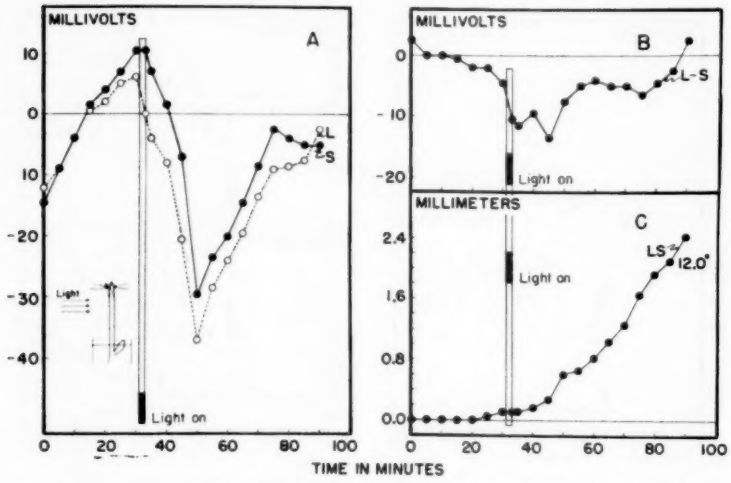

FIG. 3. Electrical and curvature responses to stimulation by 200 mcs of unilateral illumination with contact positions shown by inset. Figure 3 A shows changes in the longitudinal polarity. Curve L on the lighted side and curve S on the shaded side. Figure 3 B shows the transverse electrical polarity which was obtained by subtracting curve S from curve L in figure 3 A. Figure 3 C shows the curvature responses in the same experiment.

it was in the previous instance. In this group it again appears that the electrical changes precede the initiation of curvature.

ELECTRICAL POLARITY CHANGES WHEN THE PHOTOTROPIC RESPONSE IS NEGATIVE

In this series of experiments, consisting of three groups, coleoptiles were stimulated with 31,200 mcs of unilateral light rather than 200 mcs. According to DU BUY and NUERNBERGK (3), this dosage of light should give negative phototropic curvature.

GROUP IV.—Twelve experiments were performed in this group with the contact positions the same as in group I. In nine of these the coleoptiles curved away from the light as measured by the Judkins method. Results from these nine experiments may be divided into two general classes, early-responding and late-responding. For this reason two typical experiments are included. The difference is undoubtedly due to individual variation of

the plants involved. Only one set of curves for changes in the longitudinal polarity are plotted (fig. 4 A) because they represent the characteristic changes. The initial polarity is atypically oriented (the apex should be positive to 10 millimeters below the apex—WILKS, **13**). This reversed orientation occurs in all but three of the experiments. The explanation is that the lower contact was located either apically or basally to the region of greatest negativity.

The immediate effect of stimulation on the longitudinal polarity seems to be an increase in polarity when referred to the usual orientation of the

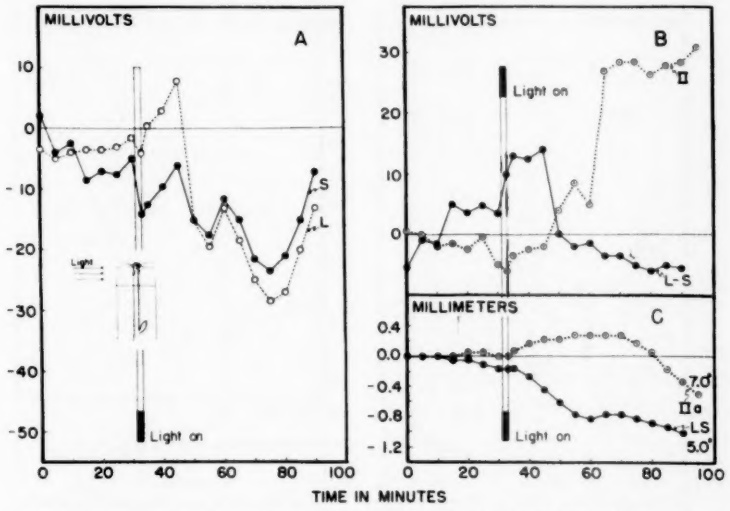

FIG. 4. Electrical and curvature responses to stimulation by 31,200 mcs of unilateral illumination with contact positions shown by inset. Figure 4 A shows changes in the longitudinal polarity. Curve L on the lighted side and curve S on the shaded side. Figure 4 B shows the transverse electrical polarities from the two experiments. Curve L–S was obtained by subtracting curve S from curve L in figure 4 A. Curve II was similarly obtained from another experiment. Figure 4 C shows curvature responses in the two experiments whose electrical responses were shown in figure 4 B.

apex positive to the basal contact region. This is followed by a longer and larger decrease in the electrical polarity, and finally there is a gradual increase. Except for the initial slight increase in polarity the electrical responses are similar to those of the corresponding group with less illumination (group I).

Two curves representing the transverse polarity changes are plotted in figure 4 B. Curve L–S was obtained from figure 4 A, and curve II was derived from a similar set of curves which are not included. In curve L–S the lighted side becomes electropositive to the shaded side soon after illumination, while in curve II the lighted side does not become electropositive to the shaded side until later in the experiment.

The curvatures of the coleoptiles whose electrical responses have just been described are shown in figure 4 C. Both curves show negative bending, but the curvature in curve LS begins earlier than in curve IIa. It seems significant that in the experiment represented by curve LS both the curvature and the transverse electrical polarity changes occur early, while in the other experiment both changes occur late. In both cases the change in the transverse electrical polarity precedes the beginning of the curvature response. It also should be noted that the bending shown by curve LS stops soon after the corresponding transverse electrical polarity returns to zero.

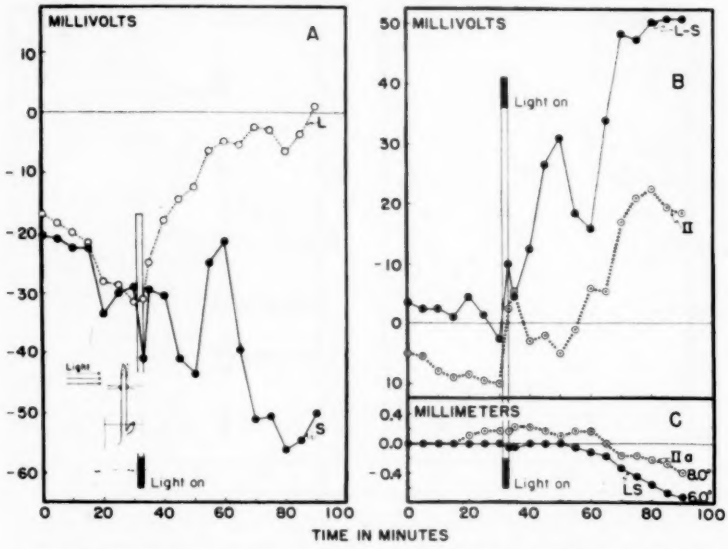

FIG. 5. Electrical and curvature responses to stimulation by 31,200 mcs of unilateral illumination with contact positions shown by inset. Figure 5 A shows changes in the longitudinal polarity. Curve L on the lighted side and curve S on the shaded side. Figure 5 B shows the transverse electrical polarities from two experiments. Curve L–S was obtained by subtracting curve S from curve L in figure 5 A. Curve II was similarly obtained from another experiment. Figure 5 C shows curvature responses in the two experiments whose electrical responses were shown in figure 5 B.

GROUP V.—Ten experiments were performed with the contacts placed in the same positions as in group II. All showed negative phototropic bending by the Judkins method. Two experiments were chosen as typical. One gave a very large transverse electrical response and the other a much more moderate one, although both responses were qualitatively similar. The longitudinal polarities of only one experiment were plotted as they were typical. The changes in the longitudinal polarity are illustrated by the curves L and S in figure 5 A. The effect of light was to increase the polarity of the shaded side (curve S). However, the opposite result is obtained on the lighted side

(curve L). In other experiments of this group, the changes in the longitudinal polarity are not quite so large but the general results are similar.

Two experiments were used to demonstrate changes in the transverse electrical polarity. These curves are found in figure 5 B. In the experiment represented by curve L–S, the lighted side immediately becomes positive to the shaded side. It continues to become more positive in surges until the end of the experiment. This curve shows a series of three peaks in the potential difference between the lighted and the shaded side. In curve II, the transverse potential is slightly less than zero but constant before stimulation. After stimulation, it follows practically the same course of events as curve L–S with three positive peaks of the lighted side.

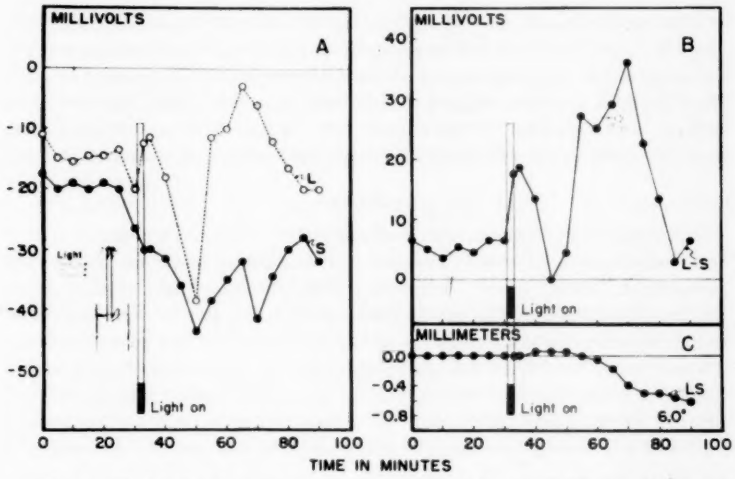

FIG. 6. Electrical and curvature responses to stimulation by 31,200 mcs of unilateral illumination with contact positions shown by inset. Figure 6 A shows changes in the longitudinal polarity. Curve L on the lighted side and curve S on the shaded side. Figure 6 B shows the transverse electrical polarity which was obtained by subtracting curve S from curve L in figure 6 A. Figure 6 C shows the curvature responses in the same experiment.

Curves LS and IIa in figure 5 C illustrate the curvature responses. For reasons previously pointed out, the slight positive movement of the tip is not a manifestation of phototropic curvature, especially since it starts before stimulation. In every experiment of this group, the time at which the lighted side becomes positive to the shaded side is much earlier than the beginning of the negative bending.

GROUP VI.—This group is composed of ten experiments of which six showed negative phototropic bending by the Judkins method. The contacts were placed as in group III. Curves representing the change in the longitudinal polarity are given in figure 6 A. The polarity is typically oriented, the apex being negative to the base. The immediate effect of stimulation

was to increase the longitudinal polarity on both the lighted and shaded sides. The changes on the lighted side are greater in magnitude than those on the shaded side.

Changes in the transverse potential difference are shown in figure 6 B. The voltage during the control period is slightly above zero, but it is constant. After stimulation the lighted side immediately becomes positive. The polarity then returns to zero and a second positivity of the lighted side occurs. It is noticed that the first and the beginning of the second positive spike of the lighted side precedes the beginning of the curvature. These results are quite typical with one exception. In the exceptional case, both curvature and the change in the transverse polarity occur in the last 10 minutes of the experiment.

The curvature response of the typical experiment is given in figure 6 C. Negative curvature does not begin until 30 minutes after stimulation. The two experiments of this group which showed bending toward instead of away from the light are interesting in that in both cases the lighted side definitely became electronegative to the shaded side. These electrical responses are precisely what one would expect from coleoptiles bending toward the light.

Discussion

Unilateral illumination, which causes either positive or negative curvature, also induces consistent changes in the inherent electrical field of the coleoptile of *Avena sativa*. Regardless of the dosage of light applied or the original orientation of the longitudinal polarity, the general tendency is for the apex to become more negative to the basal regions (the curves in figure 2 A and curve L in figure 5 A are exceptions). Uniform illumination with a dosage of about one million mcs has the opposite effect on the longitudinal polarity of the coleoptile (**1, 9**). This fact, however, does not necessarily represent a contradiction because of the large differences in the dosages of illumination.

The changes in the transverse electrical polarity in response to 200 mcs of unilateral illumination reported in this paper are not the same as were found in a previous study (**6**). Oppenoorth has published results of experiments that correspond to group III of the present study. His plants were unilaterally illuminated with 500 mcs of unfiltered mercury light. He used gelatin threads for electrical contacts. His data show that the lighted side becomes electropositive to the shaded side by about 25 millivolts and that this level was maintained for 100 minutes. Since the curvature of the Avena coleoptile to a given dosage of light varies from plant to plant, it is important to follow the curvature responses along with the electrical changes. Oppenoorth did not make continuous records of the bending process but stated that the coleoptiles did show slight curvature, presumably toward the light. He indicated that the gelatin contacts were probably too stiff to permit normal bending.

The curves in figures 1 B and 3 B, from plants that were allowed to bend toward the light without hindrance, indicate that the illuminated side be-

comes electronegative to the shaded side. This is in agreement with previous results obtained by SCHRANK (**8**), but it is opposite to OPPENOORTH'S observation (**6**). Since it is impossible to reconcile this difference in the polarity of the electrical responses on the basis of the available information, it must be left to future experimental analysis.

Unilateral stimulation by 31,200 mcs (figs. 4 B, 5 B and 6 B) always established a transverse electrical polarity opposite in orientation to that induced by 200 mcs. The orientation of the transverse electrical polarity found in the present experiments is such that the potentially convex side becomes electropositive to the opposite side regardless of whether the plant bends toward or away from the light. In only one group (fig. 2), the side destined to become convex becomes electronegative. The section of the coleoptile, which was measured for electrical changes in this group, was 10 millimeters below the apex. WENT and THIMANN (**12**) and VAN OVERBEEK (**11**) have shown that if lateral transport plays a major role in phototropism of coleoptiles, it is most likely to occur in the apical section. Therefore, the apical portion of the coleoptile, and not the basal segment, should be considered critical in postulating the transverse electrical polarity as the cause of lateral transport.

The orientation of the transverse electrical polarity, which is established in the apical region of the coleoptile, is correct to account for the electrophoretic transport of auxin as is postulated by the Went-Kögl theory. Though KOCH (**5**) has demonstrated electrophoretic movement of auxin in agar blocks, it still remains to be established whether or not a voltage of this magnitude is adequate to achieve the same result in the coleoptile cells. It is also apparent that the data herein presented do not exclude the possibility that unilateral illumination induces different rates of auxin synthesis on the lighted and shaded sides. Thus the electrical polarities could conceivably be reflecting changes in metabolism, which in turn lead to differential elongation rates.

Summary

1. Curvature and electrical responses of three Avena coleoptile segments to unilateral illumination of 200 and 31,200 mcs were observed.

2. Two hundred mcs generally caused the coleoptiles to bend toward the light and 31,200 mcs, in most cases, caused the plants to bend away from the light source.

3. The usual effect of unilateral illumination on the longitudinal polarity was to cause the apical level to become more electronegative to the basal region.

4. In the apical region of plants stimulated by 200 mcs of light the lighted side became electronegative to the shaded side. In the basal portion of coleoptiles, similarly illuminated, the lighted side became electropositive to the shaded side.

5. When the contacts were placed at the apex and at the base, and the plant stimulated by 200 mcs of light, the lighted side became electronegative to the shaded side in the apical contact area.

6. In all plants that bent away from the light, when 31,200 mcs of light were used for stimulation the lighted side became electropositive to the shaded side regardless of the position of the contacts.

7. The transverse electrical polarities in the apical contact area, which resulted from unilateral illumination, are correctly oriented to function as an electrophoretic mechanism of lateral auxin transport. However, it is also conceivable that these electrical polarities are reflecting changes in metabolism, which in turn lead to differential elongation rates and curvature.

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GENETIC AND CHLOROPHYLL STUDIES OF A YELLOW-GREEN MUTANT IN MUSKMELON

THOMAS W. WHITAKER

(WITH ONE FIGURE)

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Introduction

Chlorophyll-deficient individuals have been reported in most species of plants studied intensively by genetic methods. The majority of these chlorotic types have been shown to be the expression of a single gene mutation. However, there is a large group of types that exhibit maternal inheritance, and transmission of the deficiency to their offspring is conditioned by the cytoplasm.

Until rather recently most workers have been content to report the hereditary nature of the individual deficiencies and their end results without attempting to analyze the physiological effects upon the plant. The chief hindrance to analytical and quantitative studies of the pigments has been the laborious, complicated, and time-consuming methods for their extraction, chemical separation, and identification. Comparatively rapid and simple techniques are now available for the extraction of chlorophyll from plant tissues and the quantitative determination of the chlorophyll components a and b.

HIGHKIN (**6**) working with barley analyzed eight apparently chlorophyll-deficient types (called chlorinas) of known genetic constitution. One mutant was entirely lacking in chlorophyll b. Another had a higher total chlorophyll content than normal green plants. Two were found to be similar to normal green plants in total chlorophyll content and in the ratio of chlorophyll a to chlorophyll b. This indicates that a deficiency of pigments other than chlorophyll may determine the chlorotic appearance of some of these plants. Three others were evidently chlorotic because of low chlorophyll content, and one extremely chlorotic type was completely lacking in chlorophyll.

Various types of chlorophyll-deficient plants are of fairly common occurrence in inbred lines and segregating progenies of muskmelons, *Cucumis melo* L. The writer has studied the genetics of several of these deficiencies. The present report discusses the mode of inheritance and the physiological effects of the gene controlling one of them.

Material and methods

The yellow-green mutant, the subject of this report, was first detected in an inbred line derived from a cross between Powdery Mildew Resistant Cantaloupe no. 45 and a powdery mildew resistant complex selection.

Further experimentation has shown that the yellow-green phenotype is dependent for its expression upon a single gene. The evidence suggests that it originated as a somatic mutation.

In order to determine the quantitative and qualitative effects of the yellow-green mutant, ether extracts of the chlorophyll of both yellow-green and normal green sib plants were obtained for spectroscopic analysis. The ether extracts were made by using a combination of the methods suggested by COMAR (**1**) and GRIFFITH and JEFFREY (**5**). Plants for spectroscopic analysis were grown in the greenhouse without the use of artificial illumination. The samples were taken during February and March; in most cases they were from plants approximately six weeks old. For the extraction process, the first two or three fully expanded, mature leaves, distal to the runner tip were used.

A sample of leaf tissue weighing 4.5 to 6.5 grams was macerated in a Waring Blendor in acetone to which a small amount of calcium carbonate had been added. The mixture was then filtered and the cup and paper washed with ether to remove all traces of pigments. The filtrate was made up to a volume of 500 ml. and a 50 ml. aliquot was pipetted into a separatory funnel containing ether. The ether solution was washed thoroughly by scrubbing with distilled water 15 to 20 times. For washing, it was convenient and rapid to centrifuge the solution after the introduction of water. This caused the water layer to settle rapidly, and it could be pipetted off and discarded. Finally, the solution was made up to 50 ml. volume with ether which had been dried over anhydrous sodium sulphate.

The absorption spectra of the ether extracts were determined by the use of a Beckman spectrophotometer and the chlorophyll a and b contents of the solutions were calculated from the equations derived by COMAR and ZSCHEILE (**3, 4**).

Experimental results

DESCRIPTION OF THE MUTANT.—Yellow-green plants are easily identified by their even, light-green or yellow-green color. This color is characteristic of all the chlorophyll-bearing areas of the plant including the cotyledons. The yellow-green plants show no marked decrease in vigor or reproductive capacity as compared with their normal green sibs. In studies of leaf tissue sections from yellow-green plants, the chloroplasts appeared to be similar to those from normal green plants in number and distribution. Apparently plastids of the mutant have less pigment than those of a normal green leaf. This difference in quantity probably accounts for the visible contrast in color. The analytical data support this idea.

ORIGIN AND INHERITANCE.—The mechanics of self-pollination in *Cucumis melo* when performed artificially is somewhat different from that of other species, *e.g.*, *Lycopersicon esculentum* Mill. Most varieties of muskmelons are andromonoecious, and in selfing, the anthers of the perfect flower are seldom if ever used. Usually a staminate flower from the runner tip furnishes the pollen for the perfect flower. As a rule, perfect flowers and stami-

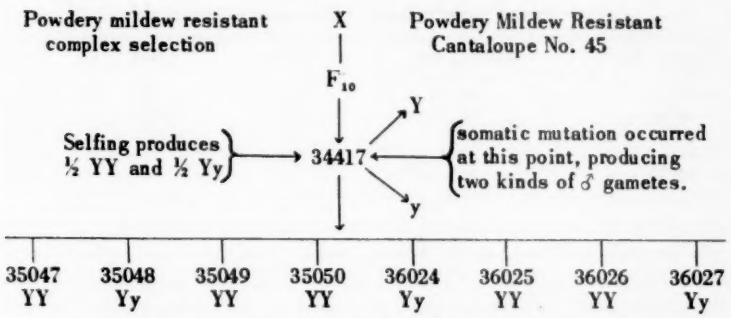

FIG. 1. Pedigree showing the origin and segregation of the yellow-green mutant.

nate flowers are on different runners. Thus a somatic mutation occurring in one runner would not necessarily affect the entire plant. This peculiarity of the muskmelon vine provides opportunity for the perpetuation of somatic mutations that might occur independently in different runners of the same plant. The yellow-green mutant evidently originated as a somatic mutation in a single runner of a homozygous green plant (YY). As a result, two kinds of male gametes were produced (Y and y). Thus when selfed, individuals with the genotype YY (green) and Yy (green) would be expected in approximately equal numbers. The same result would have occurred if the mutation had acted to produce unlike female gametes. It is suggested that the mutation occurred in the somatic tissues of plant no. 34417 (fig. 1). The progeny of this plant was normal green. Eight of the plants from this progeny were tested, and three segregated normal green and yellow-green plants when selfed (table I). Further selfing and progeny tests have supported the single gene hypothesis. The diagram (fig. 1) shows the pedigree of the yellow-green mutant, and its presumed point of origin. From the progeny of no. 35048, five green and two yellow-green plants were selfed, and their progenies were tested. The results except for the two yellow-green plants which produced only yellow-green offspring are presented in table II. The progenies of the five normal green plants segregated as expected; the

TABLE I

PROGENY TESTS OF EIGHT SELFED PLANTS FROM NO. 34417.

Plant No.	Yellow-green	Green	Total		X^2
35047	0	24	24		....
35048	60	211	271		1.18
35049	0	24	24		....
35050	0	24	24		....
36024	14	52	66		0.51
36025	0	7	7	(seed of poor viability	....
36026	0	24	24		....
36027	13	34	47		0.06

TABLE II

SEGREGATIONS FROM NORMAL GREEN PLANTS OF NO. 35048.

Progeny No.	Yellow-green	Green	Total	X^2
36041	19	51	70	0.17
36042	32	82	114	0.57
36044	12	44	56	0.38
36045	21	56	77	0.21
36046	18	49	67	0.12

χ^2 test indicates no significant deviation. The fact that all five plants were heterozygous is somewhat disturbing, and there is no obvious explanation except that an analysis of a larger population might have produced the theoretical number of homozygous green plants. However, consideration of all the data clearly suggests that the yellow-green mutant is controlled by a single gene.

TOTAL CHLOROPHYLL.—As shown in table III, the mean total chlorophyll content of single determinations from six normal green plants was 1.002 mg./gm. of fresh leaf tissue. The corresponding figure from 10 yellow-green sib plants was 0.435 mg./gm. of fresh leaf tissue. In other words, the normal green plants contain slightly more than twice as much chlorophyll as the mutant on a fresh weight basis.

Assuming that some of the normal green sib plants would have been heterozygous, we determined the total chlorophyll content of two unrelated green lines of muskmelon. The average value for these determinations was 0.635 mg./gm. fresh leaf tissue, compared with 0.269 mg./gm. for two of the yellow-green plants used as checks. This experiment was performed in July. The longer days and high temperatures probably account for the low total chlorophyll content, compared with the analyses made in February and March. However, the proportionate relation of total chlorophyll content between the yellow-green and green plants is approximately the same at the different seasons. In February and March the ratio is 1 : 2.30 and in July, 1 : 2.36. As shown above there was no significant departure in the relationship of total chlorophyll content of normal green plants with different genetic backgrounds to that of yellow-green plants. This evidence is

TABLE III

SPECTROPHOTOMETRIC DETERMINATIONS OF CHLOROPHYLL CONTENTS OF NORMAL GREEN AND YELLOW-GREEN PLANTS IN MILLIGRAMS PER GRAM OF FRESH LEAF TISSUE.

Phenotype	No. plants	Total chlorophyll	Chlorophyll a	Chlorophyll b	Chlorophyll a	Ratio a/b
					%	
Green	6	1.002 ± .06	0.709 ± .019	0.293	70.76	2.42 ± .048
Yellow-green	10	0.435 ± .024	0.331 ± .006	0.104	76.09	3.18 ± .064

interpreted to indicate that the gene responsible for green color is completely dominant over yellow-green on the basis of total chlorophyll content.

CHLOROPHYLL a AND b.—There is definite evidence of a qualitative difference in chlorophyll between the normal green and the yellow-green phenotypes. From six normal green plants the mean percentage of chlorophyll a was 70.76%, and the ratio a/b was 2.42 (see table III). On the other hand, determinations from 10 yellow-green plants yielded a value of 76.09% chlorophyll a and the ratio a/b equal to 3.18. These results indicate a qualitative difference in the chlorophyll content of the two phenotypes.

In the experiment referred to previously in which the plants were sampled in July, the chlorophyll a content of the green plants averaged 68.68% and a/b ratio was 2.19. Comparable values for yellow-green plants were 76.35% chlorophyll a, and a/b ratio of 3.23. The values obtained in this experiment suggest that chlorophyll quality in the yellow-green mutant is not altered materially by long days and relatively high temperatures.

Discussion

Observations on the yellow-green mutant through several growing seasons indicate that in spite of a reduction in total chlorophyll content and a change in the ratio of the chlorophyll components, the gene does not lower the vigor or reproductive capacity of the plants. However, leaves of normal green plants when analyzed were found to have 10.42% starch on a fresh-weight basis, comparable with 5.88% for yellow-green sib plants. These values indicate a difference in the starch manufacturing potential of the two phenotypes.

The yellow-green gene is evidently responsible for the change, in two end products, reduction in content of both a and b chlorophyll, with the relative reduction slightly greater in the b chlorophyll. The proportional reductions, however, are so similar as to suggest the possibility of both being governed by a single factor, regardless of whether one or two processes are involved.

COMAR *et al.* (**2**) reported the chlorophyll a content of two dozen species of plants including muskmelon. The mean chlorophyll a content for the 24 species was 70.52% and ranged from 67.8% for Crimean linden to 73.2% for wheat. The determinations of the chlorophyll a content for muskmelon yielded a value of 68.4%. It is evident from these studies and the previous work of WILLSTÄTTER and STOLL (**7**) that the chlorophyll a content of most species of the higher plants is approximately 70%, with some variability due to temperature, photoperiod, age of leaf, and nutrition.

Summary

A yellow-green mutant in the muskmelon (*Cucumis melo* L.) has been shown to be monogenic. There was strong evidence that it originated as a somatic mutation in a single runner of an andromonoecious plant.

The total chlorophyll content of normal green plants was slightly over

twice that of the yellow-green mutant. Furthermore, there was a qualitative difference in the chlorophyll components of the mutant, when compared with normal green plants. Thus the ratio of chlorophyll a to chlorophyll b in normal green plants was 2.42, and in the yellow-green mutant 3.18. According to the spectrophotometric data the percentage of chlorophyll a in normal green plants was 70.76 and in the yellow-green mutant 76.09.

In the heterozygous condition, yellow-green was completely recessive, and there was no marked reduction of vigor or reproductive capacity in the homozygote.

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EFFECT OF PETROLEUM OIL SPRAY ON PHOTOSYNTHESIS AND RESPIRATION IN CITRUS LEAVES[1]

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(WITH FOUR FIGURES)

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Introduction

Various formulations of petroleum fractions in the light lubricating oil range provide efficient general insecticides for control of insect pests of citrus and have been used for this purpose for many years. The relatively low cost and high insecticidal efficiency of these materials make it probable that their widespread use in citrus culture will continue for some time. Many types of injury to citrus trees have been reported, however, as resulting from such sprays.

The injurious effects of oil sprays on citrus trees have been summarized in reviews by Ebeling (**3**) and Riehl (**12**). Conclusive evidence has been presented to show that the application of oil sprays to citrus trees results in a reduction of the total percentage of soluble solids in the juice of citrus fruits. This decrease in the solids content of the juice was first noted by Yothers and McBride in 1929 (**16**). Sinclair *et al.* (**14**), Stofberg and Anderssen (**15**), and Bartholomew *et al.* (**2**) have extended the results and have shown that, when fruits from oil-sprayed trees and from non-oil-sprayed trees are compared, the use of oil spray in the usual commercial dosage causes a decrease of as much as 15% in the total soluble solids in the juice.

The relatively large portion of the citrus crop marketed at present in the form of a frozen, concentrated juice having a uniform solids content makes this lowering of the solids in the juice, resulting from oil sprays, of more importance than formerly. Since the primary constituents of the soluble solids of citrus juice are sugars and citric acid, the reduction caused by oil sprays is probably due to an interference with the net production of photosynthate by the tree, or to a decrease in the translocation of elaborated foods from the leaves to the fruit, or to a combination of these factors.

The present study was undertaken for the purpose of elucidating the effect of petroleum oils on photosynthesis and respiration of citrus leaves in the hope that knowledge concerning the mechanism of the toxic effect of oils would be useful in developing means of overcoming these effects in field practice.

Knight *et al.* (**8**) measured the effect of petroleum oils on the photosynthesis of citrus leaves by painting oil on both surfaces of the leaves of

[1] Paper no. 707, University of California Citrus Experiment Station, Riverside, California.

plants which had been starved in darkness for 15 days. Sections were cut from these leaves and tested for starch daily. It was found that when an oil having a viscosity measurement of 106 seconds was applied, the starch content of the treated leaves had not returned to normal at the end of 40 days. With an oil having a viscosity measurement of 50 seconds, the starch returned to normal on the fourth day. These same workers, using a carbon dioxide absorption-titration method, found a very marked stimulation of the respiration of citrus cuttings as a result of oil painted on the leaves. The same oil applied as a spray also stimulated respiration, but not to the same degree. The data given indicate that although the plants were illuminated, the oil application stimulated respiration so much that it was considerably in excess of photosynthesis.

GREEN and JOHNSON (**6**) found that oils containing less than 84% unsulphonated residue (U.R.) caused an increase in the respiration of bean leaves, whereas oils containing more than 84% U.R. brought about a decrease in the rates of respiration. GREEN (**5**), working with both poorly refined oil (84% U.R. or less) and highly refined oil (94% U.R. or more) on bean plants, apple leaves and twigs, and barley seedlings, found that there was, in general, an increase in the rate of respiration in response to oils supplied by atomization, but that the increase caused by the low U.R. oils was more than three times as much as that caused by the high U.R. oils. There was considerable variation within individual samples, however, and in some cases respiration was inhibited by high U.R. oil. He also found that small amounts of oil produced the same changes in the respiration of barley seedlings as large amounts of oil.

SCHROEDER (**13**), using summer spray oils applied to apple leaves, found that 1% of an oil of 80-second viscosity, 85% U.R., reduced carbon dioxide absorption 17% one to three days after oil application. The use of 2% of the same oil gave a reduction of 38.4% three to four days after application. In general, he found that inhibition of photosynthesis increased with higher viscosity and greater amount of oil applied.

HOFFMAN (**7**) applied several summer spray oils to apple leaves and found that, on the day following an application of a 1% emulsion, photosynthesis was reduced to about 20% of the rate before treatment, and that there was a gradual recovery of photosynthetic capacity. Different oils of the same type had different effects on photosynthesis. Under the conditions of his experiments, respiration occasionally exceeded photosynthesis. OBERLE *et al.* (**10**) found that, in comparison with the controls, dormant spray oils reduced the respiration of treated tissues. These workers also found a direct relation between the amount of oil applied and the inhibition of respiration.

Materials and methods

Plants of Washington Navel orange and of Eureka lemon, two important commercial species of citrus grown in California, were used in these experiments. The plants treated were rooted cuttings 18 months of age and 24 to

30 inches in height, growing in a greenhouse at a temperature of 80° F and at 50% relative humidity.

Oil treatments were made in the laboratory. The navel orange cuttings were sprayed with a laboratory sprayer operated with compressed air at a pressure of 30 pounds per square inch. The lemon cuttings were sprayed with a small-scale replica of a field spray rig operating at a pressure of 500 pounds per square inch. All the plants were given a drenching spray with full coverage of both upper and lower surfaces of the leaves. Immediately upon completion of the spray applications, the plants were returned to their former location in the greenhouse, where they remained throughout the experiment.

Plants of both citrus species were treated with an emulsion containing 2% of a California medium-grade (**4, 9**) emulsive-type spray oil. Inspection indicated that the oil deposit on the leaves was comparable in amount to that obtained under field conditions. In the experiment with navel orange three plants were sprayed with the oil mixture, and three similar plants were sprayed with water alone, to serve as controls. In the experiment with lemon six plants were sprayed with the oil mixture, and six similar plants were sprayed with water.

Determinations of photosynthesis and respiration were made with the use of a Warburg respirometer having a rectangular tank and modified to provide illumination from below the tank by means of a horizontal bank of 10 thirty-watt fluorescent lamps spaced with one eighth inch clearance between the tubes and placed 2.5 inches from the bottom of the flasks. Color quality of the light was balanced to more closely approximate sunlight by alternating Daylight (blue-white) and Warm-white (pink-white) lamps. These lamps provided an illumination of approximately 1300 fc at the bottom of the flasks.

The flasks used were of 16 ml. capacity, with two side arms. A constant carbon dioxide tension of approximately 1.5% was maintained in the system by means of diethanolamine-carbonate buffer (**11**) contained in the well and side arms. The flasks were shaken at a rate of 100 cycles per minute.

For each sampling, three leaves were harvested from the oil-treated and three from the control plants. In the experiment with orange plants, one leaf from each plant was collected at each sampling date. In the experiment with lemon plants one leaf was collected from each plant on alternate days of sampling. In both experiments, the first samples were collected 24 hours after the application of the oil spray, and sampling continued periodically until no leaves which had been oil sprayed remained on the plants.

After harvesting, the leaves were kept with their petioles in a tube of water until disk samples had been obtained. These disks were made by means of a sharpened cork borer having an inside diameter of 1 cm. Six disks were taken from each leaf, three from each side of the midrib. These disks were distributed six to a flask, so that each of the replicate flasks contained punches from all the leaves used for the treatment represented. The leaf disks were placed in the flask with the upper surfaces next to the bot-

tom of the flask, thus preventing obstruction of the stomata which occur primarily on the lower surface of citrus leaves (1). Movement of the disks during shaking was prevented by moistening the bottom surface of the flask with three drops of distilled water. By this means, overlapping of the disks and a consequent reduction in illumination was prevented.

Respiration determinations (O_2 uptake) were carried out in darkness for a total of one hour. At the end of this time the lights were turned on and the manometers were opened. After an equilibration period of 10 minutes, the system was closed, and apparent photosynthesis, as represented by the O_2 evolved, was measured for one hour. This procedure was followed by a second determination of respiration for one hour. Readings were made at 10-minute intervals, but the final data are expressed as the means of half-hourly readings for all flasks. True photosynthesis was approximated by adding the mean of pre-illumination and post-illumination determinations to the values obtained during the period of illumination. This mean of the two respiration determinations is also used as the value for respiration of the leaf disks.

Results

The effect of oil-spray treatment on the true photosynthesis of navel orange leaves is shown in figure 1. Here the rate of photosynthesis, in μl. O_2/hour/flask, is expressed as a percentage of that of the control plants. The least significant difference at the 5% level is shown by the vertical line at each point. On the first day after spray application the photosynthesis of the treated plants was slightly though not significantly higher than that of the controls. On the second day the rate of photosynthesis of the treated plants had decreased to 31% of that of the controls. With some fluctuations

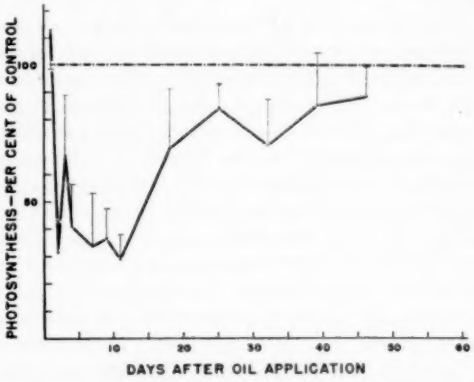

FIG. 1. Influence of oil spray on photosynthesis of Washington Navel orange leaves. Each point represents the mean of six determinations for the treated plants and is expressed as percentage of the mean of six determinations for the control plants, made at the same time. Vertical lines represent least significant differences at the 5% level.

in the relative rates, the photosynthesis of the oil-treated plants remained at a low level for at least 11 days. On the nineteenth day, the leaves had begun to recover their photosynthetic abilities. This recovery continued until the experiment was terminated at the end of 46 days, when the difference between the oxygen evolution of the two sets of plants was only 12%, or barely significant.

The rate of respiration of the same leaf samples (fig. 2) was consistently lower in the oil-treated leaves than in the controls from the second day until the nineteenth day. The next two samplings did not show statistically significant differences. In general, respiration of the leaves seems to be affected by oil in the same way as photosynthesis, but to a lesser extent. For example, it will be noted that the increased photosynthesis on the third day

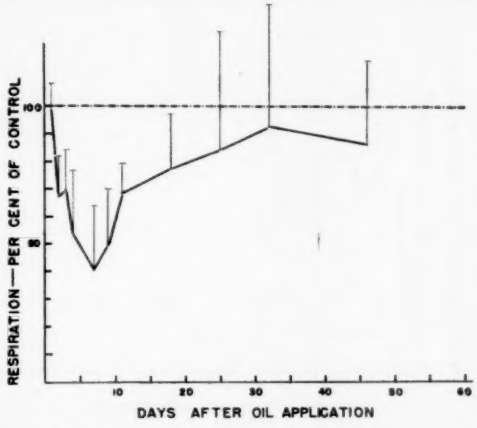

FIG. 2. Influence of oil spray on respiration of Washington Navel orange leaves. Each point represents the mean of six determinations for the treated plants and is expressed as percentage of the mean of six determinations for the control plants, made at the same time. Vertical lines represent least significant differences at the 5% level.

is reflected in an increased respiration rate for that day. This same rather close relationship between the effects of petroleum oil on photosynthesis and on respiration has been noted in other experiments in this laboratory. At no time have we found a stimulation of oxygen uptake, as was reported by KNIGHT *et al.* (8), GREEN and JOHNSON (6), GREEN (5) and others.

Oil applied to Eureka lemon plants has an effect on photosynthesis similar to that reported for applications to plants of Washington Navel orange. The photosynthesis of leaves of oil-treated plants plotted as percentage of the rate of that of leaves of control plants is given in figure 3. Comparison of these results with those obtained with orange leaves shows that the depression of photosynthesis by oil was effective more rapidly on lemon leaves, since the rate was 41% of the control on the first day after oil application.

The lowest point in the time-course of photosynthesis was reached on the second day, and on the third day the process had recovered to 68% of the control rate. From this point there was a gradual recovery of photosynthesis; but at the end of 59 days after treatment, the rate was still lower than the control, although the difference was not significant. These data, together with other similar determinations made on lemon leaves, seem to indicate that the effect of oil on photosynthesis in lemon leaves differs from its effect on photosynthesis in orange leaves in that there is a more rapid initial inhibition of the process in the former.

The respiration of lemon leaves, plotted in the same manner as the data previously given, is shown in figure 4. The effect of oil on this process in lemon leaves differs considerably from its effect in orange leaves, the initial response being slight, and even this slight effect persisting for only about 10

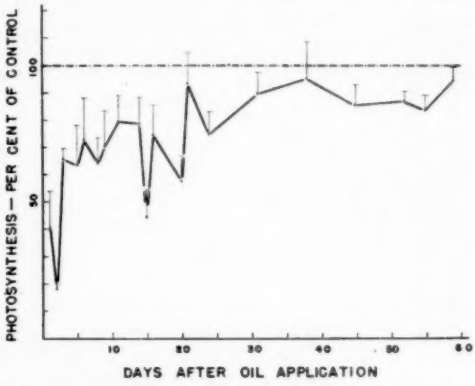

FIG. 3. Influence of oil spray on photosynthesis of Eureka lemon leaves. Each point represents the mean of six determinations for the treated plants and is expressed as percentage of the mean of six determinations for the control plants, made at the same time. Vertical lines represent least significant differences at the 5% level.

days. After 20 days there was a second downward trend which continued for approximately 20 days. After 45 days there was no longer any significant difference between the rates for the two groups of plants. This delayed inhibition of respiration has also been observed in other experiments with lemon leaves, and seems to constitute a real difference in the response of lemon and orange leaves to oil sprays.

A summary of the actual rates of true photosynthesis and respiration of the control leaves of both the Navel orange and Eureka lemon plants is given in table I. It can be seen that there was considerable variability in the photosynthesis and respiration of the leaves from day to day. Since the temperature and relative humidity of the greenhouse were controlled to approximately $\pm 5\%$, it is thought that the variation reflects differences in the light intensity to which the plants were exposed and differences in the

physiological age of the leaves selected on any one day. These fluctuations in the photosynthesis and respiration of the control plants contribute to the variability indicated by the least significant differences in figures 1 to 4. However, even though in some cases other factors were partially limiting the rates of the two processes, the presence of oil on the leaf caused an even greater depression of the rate, indicating that the relative inhibition of photosynthesis and respiration due to oil was of considerable magnitude. In fact, the inhibition was probably even greater than is indicated by the data obtained under the conditions of this experiment, where light intensity during the measurements was almost certainly a partial limiting factor.

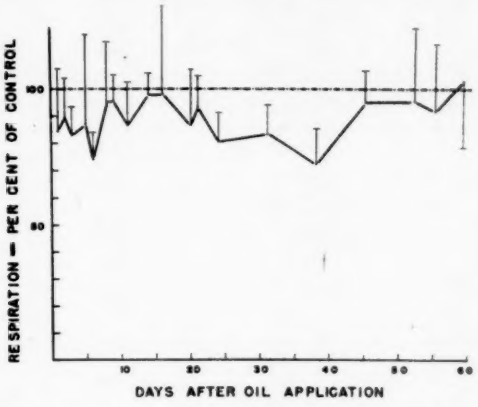

FIG. 4. Influence of oil spray on respiration of Eureka lemon leaves. Each point represents the mean of six determinations for the treated plants and is expressed as percentage of the mean of six determinations for the control plants, made at the same time. Vertical lines represent least significant differences at the 5% level.

Discussion

The data presented here, which are in general agreement with several other experiments of the same type, show that petroleum oils of the kind used as insecticides on citrus in California cause inhibition of the processes of photosynthesis and respiration in citrus leaves. The duration and magnitude of these inhibitions seem to be affected by species differences as well as by a number of environmental factors which are not clearly understood. The present experiments, which were carried out under controlled conditions in the greenhouse and laboratory, using small rooted cuttings, agree with similar determinations made of photosynthesis and respiration of trees growing in commercial groves to which petroleum oils have been applied by the usual methods. These field experiments, which are to be reported completely elsewhere, showed the same trend as was found in the work reported here, but differed in that there was more variation between the relative rates of control and oil-treated plants at different sampling rates. This

TABLE I

RATES OF TRUE PHOTOSYNTHESIS AND RESPIRATION OF UNTREATED NAVEL ORANGE AND EUREKA LEMON LEAVES.

Days	Navel orange		Eureka lemon	
	True photosynthesis	Respiration	True photosynthesis	Respiration
	$\mu l.\ O_2/cm.^2/hr.$	$\mu l.\ O_2/cm.^2/hr.$	$\mu l.\ O_2/cm.^2/hr.$	$\mu l.\ O_2/cm.^2/hr.$
1	61.6	9.6	46.1	7.7
2	62.6	11.3	57.7	8.7
3	48.2	12.0	58.1	8.7
4	76.5	12.9	....	....
5	....	....	47.0	9.5
6	....	....	59.0	8.3
7	38.5	8.0	....	....
8	50.0	7.2	....	....
9	58.0	8.6	35.8	8.0
11	47.6	8.0	37.0	8.1
14	....	....	21.4	7.2
15	....	....	28.5	4.7
16	....	....	31.2	4.7
18	35.6	4.9	....	....
20	....	....	38.3	5.5
21	....	....	40.3	8.1
24	....	....	46.4	8.2
25	44.2	5.0	....	....
31	....	....	44.7	6.9
32	59.0	6.4	....	....
38	....	....	37.9	5.8
39	61.2	7.2	....	....
45	....	....	33.2	7.6
46	44.2	8.0	....	....
52	....	....	66.1	5.9
55	....	....	68.2	6.7
59	....	....	29.3	6.1

fluctuation in the relative inhibition of both photosynthesis and respiration in the field is thought to be due to differences in environmental conditions. In experiments which are now under way, an attempt is being made to correlate these differences with various environmental factors.

The mechanism by which oil sprays affect photosynthesis and respiration in citrus remains to be determined. On the basis of present evidence, there seem to be at least two alternatives. First, there may be a direct interference with both photosynthesis and respiration, possibly due to an altered permeability of the plasma and/or other cell membranes to the passage of one or more of the gases involved. Second, there may be an indirect effect on photosynthesis caused by an accumulation of the end products of the process brought about by an inhibition of outward translocation from the leaf.

Data collected by the authors on dry weight and carbohydrate content of leaves indicate that there is an inhibition of outward transport due to the presence of oil. This type of inhibition of photosynthesis would require a

direct inhibition of respiration to prevent the stimulation of this process which would otherwise be expected from an accumulation of substrate due to inhibition of translocation. There are other possible explanations, and it seems quite likely that several factors are involved.

The question of direct respiratory inhibition has been studied by the use of disks punched from the albino portions of the leaves of variegated ivy which had been sprayed with petroleum oil emulsions. In these experiments no effect on respiration was found, but this observation is not conclusive, since substrate may have been limiting the process in the chlorotic regions of these leaves, and, in addition, the effect of oil on the respiration of this plant may be very different from its effect on citrus.

Summary

The photosynthesis and respiration of disk samples from leaves of Washington Navel orange and Eureka lemon have been studied by the use of a Warburg respirometer modified to provide illumination from a bank of fluorescent lamps placed below the flasks.

The application of petroleum oil emulsions in amounts approximating those used as insecticides on citrus was found to depress the rate of photosynthesis in leaves of both species. Some inhibition of the process persisted as long as determinations were made (59 days).

A corresponding but smaller decrease in respiration was found in these oil-treated leaves.

The data suggest that at least part of the decrease in percentages of soluble solids in the juice of the citrus fruits accompanying oil-spray applications may be due to inhibition of photosynthesis.

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THE OSMOTIC CHANGE DURING AUXIN-INDUCED WATER UPTAKE BY POTATO TISSUE

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(WITH ONE FIGURE)

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Introduction

A cardinal feature of auxin-induced growth is the absorption of water. The increase in volume accompanying cell elongation is primarily the result of the uptake of water. For this reason, it is possible to analyze the mechanism of auxin action by studying its effect on the movement of water into the cell. The auxin-induced water uptake by disks of storage tissue, discovered by REINDERS in 1938 (**10**), has been studied by a number of workers (**2, 4, 5, 6, 9, 11, 12**) with a view to furthering our knowledge of how auxin acts.

According to one theory, auxin acts by increasing the amount of osmotically active substance in the cell. This would result either in an increased internal osmotic pressure or, due to the absorption of an equivalent increment of water, in its maintenance at a constant level. For this reason, demonstration that the osmotic pressure in auxin-treated tissue is not higher than in the control tissue would not constitute evidence against this hypothesis, as was implied by VAN OVERBEEK (**12**). To test this theory of auxin action, van Overbeek made cryoscopic measurements of the osmotic pressure of sap expressed from disks of potato tissue which had been maintained in water and in auxin (α-naphthaleneacetic acid). He found that the expressed sap of auxin-treated tissues had a slightly lower osmotic concentration than that from the control tissue, and on this basis ruled out the possibility that auxin increases water uptake by increasing the amount of active solute. This conclusion was supported by similar measurements made by LEVITT (**9**).

However, the magnitude of the differences in osmotic pressure measured by these workers was such as to cast some doubt on the validity of their conclusions. VAN OVERBEEK (**12**) found the freezing-point depression of sap from auxin-treated tissues to be, on the average, 0.025° C, or about 6%, lower than that of the control, while LEVITT (**9**) reported an average difference of 0.012° C, or 1.8%. Considering the limitations of the methods employed for these measurements, conclusions based on such differences should be regarded with skepticism. On the other hand, both of the above workers reported relatively small effects of auxin on the water uptake. In van Overbeek's experiments, disks in naphthaleneacetic acid (10 mg./l.) increased in fresh weight an average of only 7.5% more than those in water, and Levitt's auxin-effect was considerably smaller. Using different methods, we have

been able to obtain a much larger auxin-induced water uptake by potato disks, and it was therefore of interest to repeat the osmotic pressure measurements under these conditions. In the present paper it is shown that when the auxin-induced uptake of water is large, there is a correspondingly large decrease in the osmotic concentration of the sap.

Methods

Water uptake

The methods employed for preparing disks of potato tissue (var. Katahdin) and for maintaining them aerobically in Petri dishes have already been described (**7**). After washing for 24 hours, the blotted and weighed disks were transferred to the experimental apparatus and water or a solution (10 mg./l.) of α-naphthaleneacetic acid (NA) was added to the appropriate level (disks breaking surface of liquid). After varying periods in a dark room at 25° C, the disks were blotted and reweighed. The water uptake could then be calculated directly.

Most of the previous work on potato disks has been done under non-sterile conditions. It appears that if the disks are thoroughly washed and kept well aerated, little exosmosis of solutes occurs (**11**) and as a result infection is very slight. Although van Overbeek (**12**) obtained a larger auxin-induced water uptake when precautions were taken to provide asepsis, his osmotic pressure measurements were made on the sap from non-sterile disks. In the experiments reported here, the very large and reproducible auxin effects obtained under non-sterile conditions made it unnecessary to work aseptically.

Osmotic concentration

After blotting and weighing, the disks were placed in large, dry test-tubes, packed in dry ice; they remained frozen overnight. All subsequent operations were performed in a 2 to 4° C cold room. The disks were removed from the tubes, and after they started to thaw (about 15 minutes) were placed (in groups of about 30 disks) in a small press-cylinder which is equipped with a perforated disk at one end and a piston, composed of a solid disk at the end of a long screw, to which pressure was applied by hand to the limit of physical ability. Broyer and Hoagland (**3**) showed that a screw press, operated by hand, gave essentially the same results as a hydraulic press. Sap expressed from the disks was collected in a centrifuge tube, centrifuged briefly, and the supernatant transferred to the inner tube of a cryoscope. Three or 4 cc. of sap (from 30 to 50 disks) were used in each determination. The jacket tube was immersed in an ice-salt bath, and a platinum stirrer and Beckmann thermometer inserted in the inner tube. The minimum and maximum (following the initiation of crystallization) temperatures were recorded to the nearest 0.01° C. The observed freezing point was corrected for undercooling using the data of Harris (**8**).

Results

The time course of water uptake by disks in water and in naphthaleneacetic acid (NA), 10 mg./l., is shown in figure 1. It is clear that the auxin-induced water uptake under the new experimental conditions is considerably larger than any previously reported effects of auxin on potato tissue. For a comparable time period (four days), and with the same auxin (NA), it is about four times as large as that reported by VAN OVERBEEK (12). Indoleacetic acid caused much smaller increases in water uptake, although values

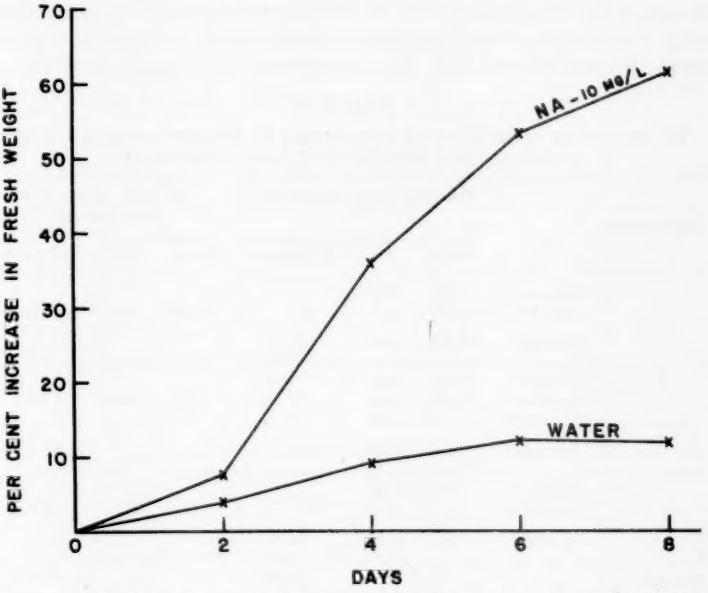

FIG. 1. The time course of water uptake by potato tissue in water and in naphthaleneacetic acid (10 mg./l.). Each point represents the average of duplicate samples, each containing 10 disks. The experiment starts when the disks are put into the apparatus, after washing for 24 hours.

with indoleacetic acid were higher than those reported by LEVITT (9). This large auxin (NA) effect is regularly reproducible although somewhat variable; in 15 experiments, the fresh weight increase after six days averaged 45%, with values ranging from 27 to 67%, for disks in NA, and 12% for those in water. This effect will be considered in more detail in subsequent papers.

The results of three experiments on the effect of NA (10 mg./l.) on the water uptake of potato tissue and on the freezing point depression of the expressed sap after four days are given in table I. The experiments began

when the disks were placed in the test solutions after washing for 24 hours. All the disks for a single experiment were cut from the same tuber. Values of fresh weight increase are means based on the weight of 50 to 100 disks. Freezing point determinations were made with the sap from 30 to 50 disks in each sample and the values of the depression are averages of two determinations which in no case differed more than 0.02° C. Corrections of the observed freezing point depressions for undercooling were never more than 0.02° C, and only corrected values are given. The amount of water absorbed by potato tissue varies somewhat from one tuber to the next, as can be seen in these results; however, the variation here is not very large. Similarly, there is a real though not great variation in the freezing point depressions. It must be noted that these variations are in the same direction.

TABLE I

THE EFFECT OF AUXIN ON THE WATER UPTAKE BY POTATO TISSUE AND ON THE FREEZING POINT DEPRESSION OF THE EXPRESSED SAP.

Experiment		Freezing point depression °C			Per cent. increase in fresh weight		
		Water	NA	Difference	Water	NA	Difference
1	Sample 1	0.54	0.41				
	Sample 2	0.49	....		11.6	35.9	+24.3
	Average	0.52	0.41	−0.11			
2	Sample 1	0.56	0.36				
	Sample 2	0.56	0.41		7.3	40.6	+33.3
	Sample 3	....	0.41				
	Average	0.56	0.39	−0.17			
3	Sample 1	0.57	0.43				
	Sample 2	....	0.43		9.0	37.8	+28.8
	Average	0.57	0.43	−0.14			
Average				−0.14			+28.8

From the data, it is clear that the osmotic pressure of sap expressed from disks kept for four days in NA is markedly lower than that from disks kept in water. The average freezing point depression difference of −0.14° C represents a decrease in osmotic pressure of 25% below the level of the control. It must be concluded that, while increasing the water uptake, auxin definitely lowers the osmotic pressure of the cell sap of potato tissue.

Discussion

The above conclusion means that the stimulation of water absorption by auxin cannot be due entirely to the formation of extra osmotically active solutes in the cell solution. In fact, if we reexamine the data of table I quantitatively, there is little or no net increase in osmotic content, as is shown in table II, in which the data have been expressed as per cent. of the

controls. The effects of auxin on fresh weight and on the freezing point depression are almost exactly equal. This could only be true if the amount of solute in the cell sap remained constant. The NA-induced lowering of the osmotic pressure appears as a result of the increase in volume. It is true that this conclusion is based on the results of only three experiments; nevertheless, the values cited are in striking agreement.

There are indications in the literature that auxin increases the rate of starch hydrolysis in various tissues. If this be the case in potato tubers, then the above conclusion would suggest that the additional sugars may be metabolized at a correspondingly greater rate. Direct measurements showing that NA does increase the respiration will be reported elsewhere. The increased starch hydrolysis caused by aeration (**1**) occurs, of course, in both controls and auxin-treated sections.

TABLE II

THE DIFFERENCES BETWEEN SECTIONS IN WATER AND IN AUXIN SOLUTION EXPRESSED AS PER CENT. OF CONTROL.

	Difference in fresh weight as per cent. of final weight of control	Difference in freezing point depression as per cent. of final depression of control
Experiment		
1	21.8	21.2
2	31.0	30.4
3	26.4	24.6

We may conclude that the auxin-induced water uptake by potato tissue and probably the more general effects of auxin on growth must be, at least in large part, the result of an influence either on the wall pressure or on an active, non-osmotic movement of water. This confirms the conclusion reached earlier by VAN OVERBEEK (**12**).

Summary

Although previous workers have reported that auxin lowers the osmotic concentration of the cell sap of potato tuber tissue, these effects have been rather small. Under new experimental conditions, a very large auxin-induced water uptake by potato disks has been obtained. Correspondingly, it has been shown that the auxin causes a large and clear decrease in the osmotic concentration (determined cryoscopically) of the expressed sap. This decrease can be accounted for by the increase in volume. It is concluded that auxin-induced water uptake, or growth, cannot be primarily the result of the formation of additional osmotically active substances in the cell.

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SURFACE ACTIVITY AS RELATED TO PHYSIOLOGICAL ACTIVITY OF PLANT GROWTH-REGULATORS

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(WITH TWO FIGURES)

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Introduction

Among theories proposed to explain the action of plant growth-regulators is one which focuses attention on the altered permeability of cell membranes caused by the adsorption of growth-controlling compound to this phase boundary. This theory, proposed by VELDSTRA (**11**), is based in part on a polarographic investigation in which the effects of the compounds on the oxygen maximum were used as a measure of the adsorbability of the compounds and therefore of their physiological activity. Subsequently (**12**), the concept was modified to allow for a chemical reaction between growth regulator and cellular constituents; and the results achieved at the surface of the mercury drop according to this hypothesis must then be considered as indicative of chemical reactivity as well as adsorption.

The possibility that an examination of substances with respect to their effect on the oxygen maximum might indicate a relative measure of their adsorbability or surface activity was first propounded by Heyrovsky and detailed by RASCH (**5**). According to this theory, the oxygen maximum is the result of the adsorption of oxygen to the surface of the mercury drop, and any reduction in this maximum (at the same potential) is due to the preferential adsorption of some other substance which replaces oxygen at the mercury surface.

Another explanation of the maximum involves the electrokinetic stirring in the mercury drop and the electrolyte solution (**7**) which brings reducible ions to the mercury surface more rapidly than diffusion alone would. At the maximum potential the density of indifferent cations in the electrical double layer restricts the movement of reducible ions to that of diffusion, whereupon the current drops to the diffusion current. Surface active substances depress the maximum, according to this theory, by lowering the surface tension of the mercury and the subsequent cessation of the stirring effect. According to KOLTHOFF and LINGANE (**2**) neither one of the theories adequately accounts for all characteristics of the maximum but the complete explanation probably will include aspects of both.

The number of compounds reported on by Veldstra was few, and the growth tests with which the results were compared were the rooting of cuttings and the curvature of split stems of peas. It was thought that a more comprehensive investigation of the effects on the oxygen maximum of compounds effecting cell elongation together with several compounds known to

be inactive in this respect would throw more light on the relationship between surface activity and physiological activity.

Materials and methods

Following the procedures of VELDSTRA (**11**) as closely as possible, a solution of the growth regulator was made up with conductivity water (as were all solutions used in this work) in a concentration usually of 1×10^{-3} mole per liter. This solution was added in aliquots of 0.01 ml. each to the electrolyte solution which consisted of 10 ml. of a mixture of 5 ml. of methyl alcohol with 45 ml. of 0.01 *N* potassium chloride, the resulting solution of growth regulator being 1×10^{-6} mole per liter. In several cases the oxygen maximum was either too greatly or too slightly suppressed by the concentration employed; and when possible, either a more dilute or a more concentrated solution was used. Additions of some compounds therefore produced increments in molar concentrations of 0.1×10^{-6}, 5×10^{-6}, or 1×10^{-5} mole instead of 1×6^{-6} mole. The number of aliquots added to the test solution was limited to about 10 to prevent dilution effects in the determination. The control in all cases was run on the electrolyte solution without the growth regulator. Since the slightest bit of extraneous material exerts a considerable influence on the height of the oxygen maximum, all glassware was acid washed with sulphuric acid and potassium dichromate and care was taken to see that none of the cleansing solution was left to interfere with the determination.

Introduction of the growth regulator into the solution causes a lowering of the height of the maximum, and the amount of growth regulator that will depress the height to one half of its value in the original electrolyte solution is designated as the half-suppression value (H.S.V.) for that compound. The H.S.V. is always expressed in micromoles per liter. As an example, the determination of the H.S.V. for the compound, *p*-methoxyphenoxyacetic acid, is shown in figure 1. The first curve of the polarogram is the control which is obtained for the electrolyte solution free of growth regulator. The height of the maximum is the distance AD. The distance AB indicates the amount of suppression achieved with one addition of the solution of the growth regulator producing a molar concentration of 1×10^{-6}. The distance BC indicates the suppression caused by another addition. Since the distance AC is one half the distance AD, the concentration of the solution after the second addition of the growth regulator is 2×10^{-6} mole per liter and the H.S.V. is 2.

A high-speed, photographic recording Polarograph (Sargent Model XII) was employed in these investigations. The electrolysis vessels used have the Sargent number, S-29305. The span electromotive force was 4 volts, but the range of voltage change used was from 0 to 0.8 volt since the oxygen maximum occurs at approximately 0.4 volt. The shunt ratio (which controls the sensitivity) was 50, and the temperature was maintained at $25 \pm 0.05°$ C by means of a thermostatically controlled water bath. The drop

time for the capillary electrode used in Veldstra's investigations was one half second per drop which is rather fast. In fact, KOLTHOFF and LINGANE (**2**) in their work on diffusion currents have found that a drop time of from three to six seconds per drop will, in general, give the most reproducible results. VARASOVA (**10**), and RAYMAN (**6**) found that a quickly dropping mercury electrode (one half second per drop) is less sensitive to additions of adsorbable substances than a more slowly dropping one. With a fast drop time more of the test solution must be added to the electrolysis vessel before a half suppression value is obtained. Moreover, in the present work, it was found that a drop time of 7.2 seconds per drop increased the maximum and therefore this slower drop time was used. The results with this drop time were entirely reproducible.

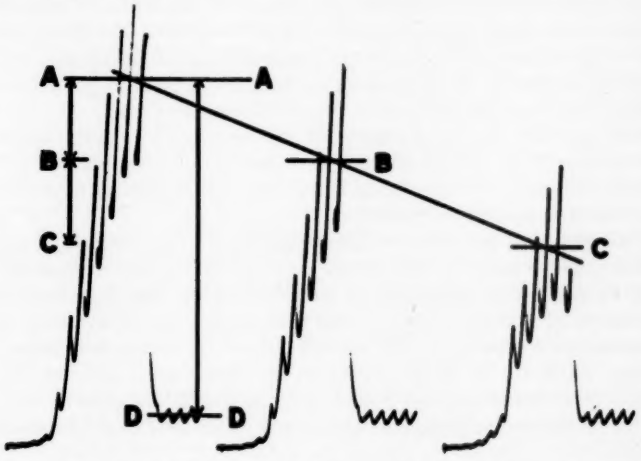

FIG. 1. The determination of the half-suppression value for *p*-methoxyphenoxyacetic acid. See text for explanation.

Most of the compounds which were tested had been prepared and their purity ascertained by Dr. Corwin Hansch of the Department of Chemistry of Pomona College, Claremont, California. Other compounds, obtained from Eastman Kodak Company, were purified by successive recrystallizations and checked for melting points by Dr. Hansch.

The physiological activity of the compounds which is compared with their surface activity in this investigation is determined by their effect on the elongation of portions of Avena coleoptiles relative to the effect of indoleacetic acid (**1, 3, 4**).

Experimental results and discussion

VELDSTRA has suggested (**12**) that the molecule of a growth regulator which has maximal activity has both a physico-chemical and a spatial rela-

tionship between the polar and non-polar parts of the molecule. Thus the molecule has a lipophilic part in balance with the hydrophilic carboxyl group, and substitutions in either portion of the molecule will bring about changes in the hydrophilic-lipophilic balance which will in turn influence the physiological activity of the substance. As an example, the addition of a chlorine atom in the para position of phenoxyacetic acid makes the substance more lipophilic, and its physiological activity also increases; but if three chlorine atoms are added to the phenoxyacetic acid in positions 2, 4 and 6, the compound is made too lipophilic, the balance is destroyed, and its physiological activity decreases.

In this concept the effects of the growth regulator at the surface of separation of mercury and water and the boundary surface in the living cell depend on the hydrophilic-lipophilic balance in the molecule. The action of the growth regulator is further divided into two steps. The first reaction involves the adsorption of the growth regulator to a cell membrane, and the second takes place in the protoplasm of the cell and involves a combination of the growth regulator with one or more cellular constituents. In the first reaction, varying degrees of adsorption are postulated. Adsorption which is too strong throws off the physiological balance between the cell and its environment; and if the adsorption is too slight, the conditions necessary for greater growth will not be realized.

If adsorption is necessary for and proportional to the action of a growth regulator, and relative surface activity or adsorption can be measured, it should be possible to determine the balance between lipophilic and hydrophilic parts of a molecule and in this way predict the growth activity of substances as yet untested. On the other hand, if suppression values are obtained which are out of proportion to the physiological activity of substances, or if compounds are found to be active in the growth test but inactive in suppressing oxygen maxima, the hypothesis should be considered invalid.

A comparison of half-suppression values reported (**11, 12**) for several compounds and the values obtained in this investigation together with the physiological activity of the compounds in the pea test as reported by THIMANN (**8, 9**) and their activity in the Avena section test at 10^{-5} M concentration is presented in table I. The lack of agreement of the half-suppression values as determined with those reported by Veldstra is attributed to differences in technique which may be of several types. It may be noted that Veldstra reported the half-suppression value for naphthaleneacetic acid as 9 in 1944 and 5 in 1949, whereas the value found in this investigation was 0.4.

Veldstra's hypothesis would appear to be borne out if the relative physiological activities of the compounds in the pea test and his half-suppression values are compared. Such an examination would seem to indicate that a certain hydrophilic-lipophilic balance between the parts of the molecule characterizes the most active growth regulator and that compounds ap-

TABLE I

A COMPARISON OF HALF-SUPPRESSION VALUES FOR VARIOUS COMPOUNDS AND THEIR PHYSIOLOGICAL ACTIVITY AS SHOWN BY THE PEA TEST AND THE AVENA SECTION TEST.

Compound	Relative physiological activity		Half-suppression values	
	Pea test (8, 9)	Avena section test (10^{-5} M)	Reported (11, 12)	Found
Indoleacetic acid	100	100	11	0.65
Phenylacetic acid	0	50	1100	10.0
Phenoxyacetic acid	ca. 0	5	130	13.0
o-Chlorophenoxyacetic acid	4	5	17	4.0
m-Chlorophenoxyacetic acid	....	30	....	2.5
p-Chlorophenoxyacetic acid	200	40	14	2.5
2,4-Dichlorophenoxyacetic acid	1000–1200	85	7	2.0
2,4,5-Trichlorophenoxyacetic acid	500	85	....	0.75
2,4,6-Trichlorophenoxyacetic acid	0.4	0	4.8	1.0

proaching this optimal balance show increased activity, and in general, a lower half-suppression value. Those molecules such as *o*-chlorophenoxyacetic acid, phenoxyacetic acid and phenylacetic acid with more hydrophilic portions are reported as inactive in the pea test as is 2,4,6-trichlorophenoxyacetic acid which has a more lipophilic portion. However, if the physiological activity in the Avena section test is examined, it is apparent that phenylacetic acid is quite active although it has a very high half-suppression value and is rather hydrophilic in nature, while *p*-chlorophenoxyacetic acid with a lower half-suppression value and a more lipophilic character is less active.

It is also evident from table I that 2,4,5-trichlorophenoxyacetic acid, with a surface activity similar to that of 2,4,6-trichlorophenoxyacetic acid, displays high physiological activity. Although the surface activity is not greatly altered by shifting the chlorine atom from the 5 position to the 6 position, the physiological activity is completely nullified. In the compounds listed in table I, there is no correlation between the degree of physi-

TABLE II

HALF-SUPPRESSION VALUES AND PHYSIOLOGICAL ACTIVITIES OF SEVERAL DERIVATIVES OF PHENOXYACETIC ACID.

Compound	Physiological activity	Half-suppression value
o-Methylphenoxyacetic acid	Active	4.0
p-Methylphenoxyacetic acid	Active	2.5
3,5-Dimethylphenoxyacetic acid	Active	0.9
2,4,6-Trimethylphenoxyacetic acid	Inactive	3.0
2,4-Dibromophenoxyacetic acid	Highly active	0.5
2,6-Dibromophenoxyacetic acid	Inactive	1.0
2,4,6-Tribromophenoxyacetic acid	Inactive	0.4

TABLE III

HALF-SUPPRESSION VALUES AND PHYSIOLOGICAL ACTIVITIES OF DERIVATIVES OF BENZOIC ACID.

Compound	Physiological activity	Half-suppression value
o-Hydroxybenzoic acid	Inactive	5.0
o-Aminobenzoic acid	Inactive	8.0
o-Chlorobenzoic acid	Active	over 140.0
m-Chlorobenzoic acid	Inactive	20.0
2,4-Dichlorobenzoic acid	Inactive	30.0
2,6-Dichlorobenzoic acid	Active	over 20.0

ological activity in the Avena section test and the half-suppression values determined in this investigation.

A further divergence between the expected results and those obtained for the other phenoxyacetic acid derivatives is shown in table II. The physiological activity of the first three methyl-substituted phenoxyacetic acids in the Avena section test is fairly uniform yet 3,5-dimethylphenoxyacetic acid exhibits much more suppressive action on the oxygen maximum than the other two compounds. Although 2,4,6-trimethylphenoxyacetic acid has a half-suppression value which is intermediate, it has no physiological activity. The bromine derivatives display relatively uniform half-suppression values although 2,4-dibromophenoxyacetic acid is highly active, while 2,6-dibromophenoxyacetic acid and 2,4,6-tribromophenoxyacetic acid are inactive.

The half-suppression values and physiological activities of benzoic acid derivatives are given in table III. The surface activities of these compounds show large variations, yet those with the greatest suppressive action are completely inactive in the Avena section test and the compounds bringing about elongation have very little suppressive action. Moreover, *ortho*-chlorobenzoic acid has almost no surface action and yet effects the elongation of Avena coleotile sections. As can be seen in figure 2, the additions of 2,6-dichlorobenzoic acid have practically no effect on the oxygen maximum. The additions were made in 0.01 ml. aliquots of a solution with a molar concentration of 5×10^{-3} which resulted in increments of 5×10^{-6} mole per liter with each addition. The compound is sparingly soluble in water,

TABLE IV

HALF-SUPPRESSION VALUES AND PHYSIOLOGICAL ACTIVITIES OF INDOLEACETIC ACID AND SEVERAL DERIVATIVES.

Compound	Physiological activity (10^{-4} M)	Half-suppression value
Indoleacetic acid	100	0.65
4-Chloroindoleacetic acid	80	0.3
2-Methylindoleacetic acid	45	0.4
4,7-Dichloro-2-methylindoleacetic acid	0	0.4

and an examination of the effects of a more concentrated solution on the maximum was not possible using the general procedure employed for the other compounds.

In table IV the half-suppression values and physiological activities of indoleacetic acid and several derivatives are compared. In this group of compounds the surface activity was found to be remarkably uniform with half-suppression values ranging from 0.3 to 0.65 while the activity in the Avena section test varies greatly. One of the derivatives, 4,7-dichloro-2-methylindoleacetic acid, is inactive at 10^{-5} M while 2-methylindoleacetic acid has approximately half the activity of 4-chloroindoleacetic acid which in turn is nearly equal to that of indoleacetic acid although its half-suppression value is only half that of indoleacetic acid.

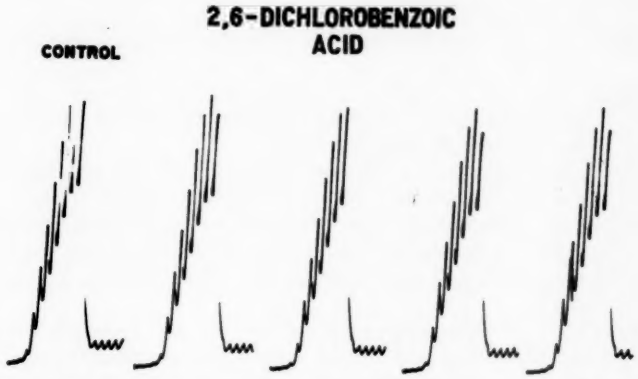

FIG. 2. Polarogram of the oxygen maximum in the control electrolyte solution without growth regulator and the oxygen maxima obtained following additions of one, two, three, and four 0.01 ml. aliquots of 5×10^{-3} M 2,6-dichlorobenzoic acid.

Although the half-suppression values do not indicate the growth activities of the various compounds, it is still possible that they measure the adsorption of the compounds at a boundary surface which Veldstra proposes as the first step in the action of the growth regulators. If such an adsorption is involved in the growth reaction, the chemical reactivity determined by molecular structure must be more important than the hydrophilic-lipophilic balance of the growth-regulating molecule.

Summary

The suppressive effect of plant growth-regulators upon the polarographic oxygen maximum has been used as a measure of the activity of these substances at a phase boundary. This suppressive effect has been determined for 25 compounds including substances which promote the straight growth of Avena coleoptile sections and substances which are inactive in this respect. There is no correlation of surface activity measured in this way with

physiological activity. In contradiction of the hypothesis that a pronounced effect in suppression of the oxygen maximum is a characteristic of plant growth-regulators, 2,6-dichlorobenzoic acid exerts little if any suppressive effect upon the oxygen maximum, yet has considerable physiological activity.

It is concluded that chemical reactivity is more important than adsorbability for the manifestation of growth-regulating properties by organic compounds.

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METABOLISM OF 2,4-DICHLOROPHENOXYACETIC ACID. I. $C^{14}O_2$ PRODUCTION BY BEAN PLANTS TREATED WITH LABELED 2,4-DICHLOROPHENOXY-ACETIC ACIDS

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(WITH FOUR FIGURES)

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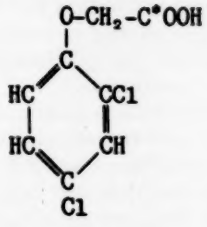
Although the morphological and physiological responses of plants to 2,4-dichlorophenoxyacetic acid (2,4-D) have been extensively studied, virtually nothing is known concerning the chemical reactions of this substance *in vivo* particularly as related to its biological effects. Evidence has been obtained by two different methods that a substantial proportion of the 2,4-D applied to a bean seedling can be recovered therefrom in apparently unchanged form (**3, 7**), but the possibility must be recognized that the recovered growth-regulator may represent only the excess beyond the amount that actually contributes to the reactions resulting in the observed developmental responses.

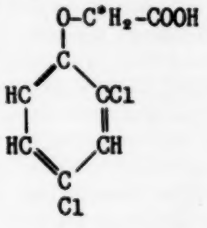
As the initial phase of a study of the transformations of C^{14}-labeled 2,4-D in higher plants, attention was directed to the production of radioactive carbon dioxide since such information would be required in the eventual formulation of a C^{14} balance sheet and might also be expected to contribute to understanding of the pathway of metabolism. The present paper describes the results of these experiments. Subsequent reports will deal with other metabolic products.

Experimental procedure

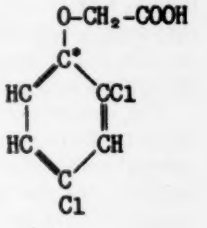
The compounds used were 2,4-dichlorophenoxyacetic acid-1-C^{14}, 2,4-dichlorophenoxyacetic acid-2-C^{14}, and 2,4-dichlorophenoxy-1-C^{14}-acetic acid (hereafter abbreviated as 2,4-D-1-C^{14}, 2,4-D-2-C^{14}, and 2,4-D-R-C^{14}) with specific activities of approximately 1, 0.5, and 0.02 mc./mmole, respectively.



2,4-D-1-C^{14}



2,4-D-2-C^{14}



2,4-D-R-C^{14}

The melting points, distribution on paper chromatograms, and physiological activities, as determined by bioassay (**1**), were identical with those of ordinary 2,4-D.

A modification of the gas counting system of JANNEY and MOYER (**4**) was used for measurement of activities. A stainless steel ionization chamber was found preferable to the brass chamber employed in the original instrument because of its apparent freedom from memory effects. Measurements were made at one atmosphere CO_2 pressure, 10 millimoles of gas being required to fill the chamber. With small samples, ordinary CO_2 was added to bring the total pressure to this value. Of a large number of experiments only those results in which the measured activities were at least four times the background values are reported here.

The general experimental procedure consisted of applying labeled 2,4-D to young bean plants (*Phaseolus vulgaris* var. Black Valentine) or parts thereof, collecting the evolved CO_2 in alkali, liberating CO_2 from an aliquot of the carbonate, and determining its radioactivity. The 2,4-D was applied by placing on the plant a 0.005 ml. drop of a solution of the desired concentration in 95% ethanol containing 1% Tween-20. During the period of collection of CO_2, the roots, or basal portions of the stems in the case of derooted plants, were immersed in a 0.01 M NaH_2PO_4 solution in order to minimize retention of CO_2.

Results

Since one of the objectives of these experiments was to obtain information which would be applicable to plants treated under greenhouse conditions, those conditions were simulated as closely as practicable. It was desired also to employ the smallest analytically feasible dosages of 2,4-D in view of the very great sensitivity of beans to this compound (**1**). The seedlings were cultured in the greenhouse to a stage just prior to unfolding of the first trifoliolate leaf from the bud. The roots were carefully washed free of soil and placed in NaH_2PO_4 solution. The 2,4-D was applied to the terminal buds and the plants were placed in a 28-liter glass jar which was sealed with a sheet of glass provided with inlet and exit tubes. The plants were illuminated from above by a bank of Daylight fluorescent lamps giving 900 fc at the primary leaves. Air from the jar was dispersed into fine bubbles by a fritted glass disk and passed first through an absorbing tower containing alkali and then through a barium hydroxide solution to verify completeness of absorption of CO_2. The entire apparatus was constructed in duplicate so that two sets of plants could be utilized concurrently.

The time course of $C^{14}O_2$ production from the side-chain is shown in figure 1. During the first 24-hour period a much greater fraction of the carboxyl carbon than of the methylene carbon is converted into CO_2. Subsequently, the rate of evolution from carboxyl diminishes while that from methylene increases. The total production of $C^{14}O_2$ from the methylene carbon during the first few days after treatment is considerably less than

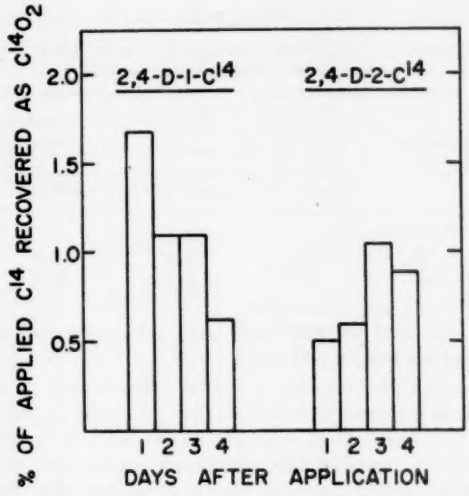


FIG. 1. Time course of $C^{14}O_2$ production by entire bean plants from side-chain-labeled 2,4-D. Dose, 1.5 μg. per plant, applied on terminal bud; light intensity, 900 fc; photoperiod, 14 hours; and ventilation rate, 14 liters per hour (Expt. 1098).

that from the carboxyl carbon. Data from seven additional experiments are presented in table I, from which it is seen that the fraction of the applied activity recoverable as CO_2 is not greatly influenced by conditions of illumination or by a sevenfold increase in dose. Experiment 818 was carried out in continuous darkness and experiment 979 in continuous light. Experiments 913, 930, and 857 were carried out under a photoperiod of 12 hours with normal air supplied during the light period and CO_2-free air during the dark period. Experiment 1097 was performed with normal air and a photoperiod of 14 hours. For the eight experiments the average per-

TABLE I

PRODUCTION OF $C^{14}O_2$ FROM LABELED 2,4-D APPLIED TO TERMINAL BUDS OF INTACT BEAN PLANTS.

Expt.	2,4-D applied per plant		Collection period	Per cent. of applied activity recovered as CO_2 from		mμmoles $C^{14}O_2$ evolved per plant from	
				2,4-D-1-C^{14}	2,4-D-2-C^{14}	2,4-D-1-C^{14}	2,4-D-2-C^{14}
	μg.	*mμmoles*	*days*				
818	1.5	6.8	3	5.1	0.7	0.34	0.048
913	1.5	6.8	4	6.8	1.4	.46	.095
930	1.5	6.8	4	5.3	1.0	.36	.068
979	1.5	6.8	4	6.2	1.1	.42	.075
857	1.5	6.8	5	7.2	2.2	.49	.15
1097	10.0	45.3	4	4.5	2.9	2.04	1.31

TABLE II

PRODUCTION OF $C^{14}O_2$ FROM LABELED 2,4-D APPLIED TO PRIMARY LEAVES OF INTACT BEAN PLANTS.

Expt.	2,4-D applied per plant		Per cent. of applied activity recovered as CO_2 from		mμmoles of $C^{14}O_2$ produced per plant from	
			2,4-D-1-C^{14}	2,4-D-2-C^{14}	2,4-D-1-C^{14}	2,4-D-2-C^{14}
	μg.	*mμmoles*				
1096	10	45.	4.4	2.8	2.0	1.3
1082	100	453.	2.9	1.2	13.0	5.5

centage of the applied activity recovered as CO_2 per day was 1.5 from 2,4-D-1-C^{14} and 0.4 from 2,4-D-2-C^{14}.

In order to ascertain whether a similar result would be obtained with still larger doses, recourse was had to leaf applications inasmuch as the sensitivity of the plant to leaf treatment is considerably lower than to bud application (**1**). A drop of 2,4-D solution was placed upon each of the primary leaves of the plant; otherwise the procedure was as already described. The data presented in table II were obtained with a collection period of three days at a ventilation rate of 14 liters of normal air per hour and a light intensity of 900 fc. Experiment 1096 was conducted with a photoperiod of 14 hours and 1082 with a photoperiod of 10 hours. The results of experiment 1096 (table II) and experiment 1097 (table I) indicate that, for a 10 μg. dose of 2,4-D, the same fraction is converted to CO_2 whether appli-

TABLE III

PRODUCTION OF $C^{14}O_2$ FROM LABELED 2,4-D APPLIED TO PRIMARY LEAVES OF BEAN PLANTS IN UNVENTILATED CONTAINERS.

Expt.	2,4-D applied per plant		Collection period	Per cent. of applied activity recovered as CO_2 from		mμmoles $C^{14}O_2$ evolved per plant per day from	
				2,4-D-1-C^{14}	2,4-D-2-C^{14}	2,4-D-1-C^{14}	2,4-D-2-C^{14}
	μg.	*mμmoles*	*days*				
519*	0.3	1.4	4	3.5	....	0.012	....
588†	0.5	2.3	2	2.1	....	.024	....
519*	1.0	4.5	4	3.0	....	.034	....
579**	1.0	4.5	3	1.5	....	.022	....
519*	3.0	13.6	4	2.4	0.8	.082	0.027
374*	5.0	22.6	6	4.1	....	.154	....
534*	5.0	22.6	2	....	0.2	....	.023
588†	5.0	22.6	2	1.1	0.4	.124	.045
498*	10.0	45.3	5	4.1	1.2	.369	.109
579**	10.0	45.3	3	1.2	0.5	.180	.076

*Continuous darkness.
**14-hr. photoperiod; intensity = 800 fc.
†14-hr. photoperiod; intensity = 800 fc, derooted plants.

cation is made to the leaf or to the bud; with a 100 μg. dose, a somewhat smaller fraction appears as CO_2.

In table III are listed results from several other trials conducted in unventilated desiccators or bell jars in which large surfaces of the absorbing alkali were exposed. Under these conditions of possible oxygen deficiency, the rate of conversion of 2,4-D to CO_2 appears to be appreciably smaller than in the ventilated cultures. During the collection periods of two to five

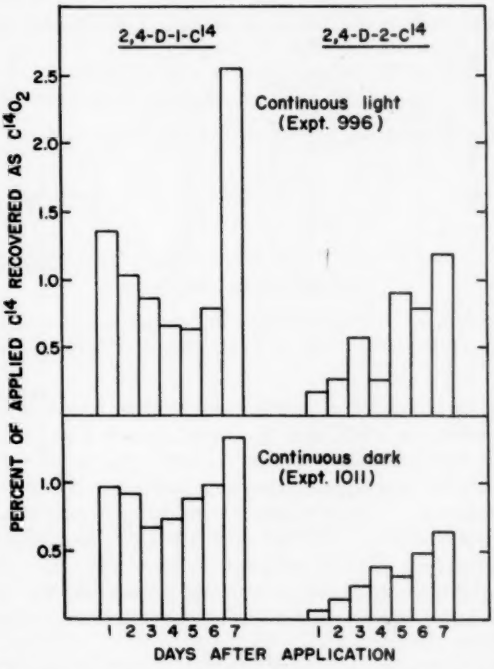


FIG. 2. Time course of $C^{14}O_2$ production by bean explants from side-chain-labeled 2,4-D. Dose, 1.5 μg. per plant, applied on terminal bud; light intensity, 900 fc; ventilation rate, 14 liters per hour.

days, the rate of conversion of the carboxyl was, in these tests also, about three times as great as that from the methylene carbon.

Owing to the large amount of $C^{12}O_2$ produced by intact bean plants relative to the amount of $C^{14}O_2$ derived from 2,4-D and to the consequent low specific activity of the total CO_2, the smallest doses which it was feasible to employ were larger than those required to elicit a marked morphological response. In order to study smaller doses, use was made of explants, consisting of 5 to 10 cm. of the stem bearing the terminal bud and cotyledons but lacking roots and primary leaves. The buds of such explants when treated

with 2,4-D developed leaves exhibiting the characteristic formative malformations (**1**); but much less CO_2 was evolved per explant than from intact plants, and many more could be accommodated in the respiration chambers.

The time course of $C^{14}O_2$ production in two experiments with such material is shown in figure 2. As with the entire plants, the rate of CO_2 production from the carboxyl carbon tends to diminish during the first few days while that from the methylene carbon increases. Long term experiments with explants have the difficulty that treatment with 2,4-D aggravates the

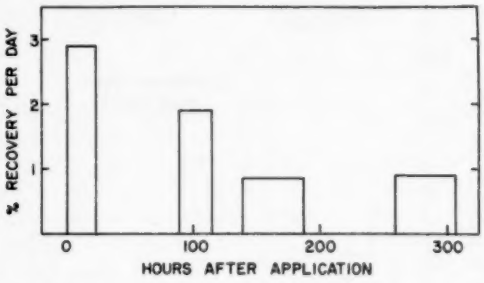


FIG. 3. Time course of $C^{14}O_2$ production by bean explants from 2,4-D-1-C^{14}. Dose, 0.04 μg. per plant, applied on terminal bud; light intensity, 900 fc; photoperiod, 14 hours (Expt. 652).

susceptibility to attack by microörganisms so that growth of molds on the tissue is frequently apparent after five or six days at high humidities. The rising rate of $C^{14}O_2$ evolution during the latter part of the experiment is probably related to this circumstance as is indicated by the experiment illustrated in figure 3. Treated explants were enclosed with alkali for a day, after which the containers were left open for a two day period, the alkali having been removed and the radioactivity measured. The explants were then enclosed with fresh alkali for a second period, and the alternation of opening and closing was repeated twice more, four fresh portions of absorb-

TABLE IV

RELATION BETWEEN DOSE OF 2,4-D-1-C^{14} AND AMOUNT OF $C^{14}O_2$ PRODUCED BY BEAN EXPLANTS.

2,4-D applied per explant		Number of experiments averaged	$C^{14}O_2$ production	
			mμmoles/explant/day	Per cent. of applied dose recovered/day
μg.	*mμmoles*			
0.04	0.18	2	0.0053	2.95
.10	.45	1	.0071	1.57
.15	.68	5	.0110	1.62
.20	.91	1	.0085	0.93
1.5	6.8	8	.0720	1.06
2.0	9.1	2	.0673	0.74
4.6	20.8	1	.125	0.60

ing solution being used in all. In this fashion it was possible to maintain the explants reasonably free of mold for 13 days and in large measure to prevent the increase in rate of $C^{14}O_2$ production. The total recovery of C^{14} following application of 0.15 μg. carboxyl-labeled 2,4-D per bud was 10.4% and, as the time of actual collection amounted to only 47% of the total duration of the experiment, it may be estimated that approximately 22% of the applied C^{14} escaped from the plants as CO_2.

With increasing doses of 2,4-D, the total amount of $C^{14}O_2$ evolved also increases but the fraction converted to CO_2 tends to diminish. Table IV summarizes the results of 20 experiments with explants using collection periods of two to four days. These data are plotted in figure 4, together with the dose-response curve for repression of leaf expansion (**1**).

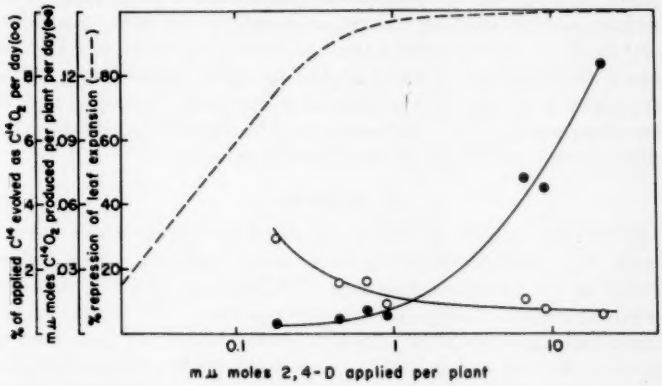


FIG. 4. Formative activity (leaf repression) and $C^{14}O_2$ production from 2,4-D-1-C^{14} as functions of applied dose of 2,4-D.

Owing to the relatively low specific activity of the available ring-labeled 2,4-D, it was necessary to employ much higher doses of this substance compared with the chain-labeled compounds. In a series of experiments with doses up to 200 μg. applied to leaves or buds of entire plants or to explants, no radioactivity was found in the evolved CO_2 during collection periods of two to six days, whence it may be concluded that less than 0.2% of ring carbon atom 1 was liberated as CO_2.

Discussion

During the course of these experiments, two reports of the formation of CO_2 from 2,4-D have appeared. The first, by HOLLEY *et al.* (**3**), states that radioactive CO_2 is produced by plants treated with 2,4-D-1-C^{14} but provides no data. The second, by FANG *et al.* (**2**), describes a single experiment in which bean plants treated with 100 μg. 2,4-D-2-C^{14} (50 μg. on each primary leaf) evolved in three days an amount of $C^{14}O_2$ corresponding to

17.5% of the applied C^{14}. So far as can be judged, this experiment was very closely similar to our Expt. 1082 (table II) in which only 1.2% of the applied C^{14} was liberated as CO_2. While this discrepancy may be due to some unrecognized diversity in plant material or environmental conditions, a difference in analytical methods also may be responsible. The technique employed by Fang *et al.* was not described in detail, and no information was given concerning the ratio of net counting rate to background so that it is difficult to assess their activity data which were obtained by extrapolation to an infinitely thin sample, a method involving considerable uncertainty (**5**).

Whether the production of CO_2 from 2,4-D is related to the physiological action of the growth regulator cannot be ascertained from the present results. It is suggestive, however, that the fraction converted to CO_2 is relatively independent of the amount of applied 2,4-D in the range of supramaximal doses but tends to rise rather sharply in the range of submaximal doses, figure 4. On the other hand, the data in tables II and III demonstrate a decomposition of 2,4-D applied to nearly mature leaves although these organs do not themselves manifest morphological responses and, in the experiments carried out in darkness, would be expected to export very little of the growth regulator to the meristematic cells (**6**).

Summary

Radioactive carbon dioxide is produced by plants which have been treated with 2,4-dichlorophenoxyacetic acids containing C^{14} in either the carboxyl or the methylene positions. The evolution of $C^{14}O_2$ continues at a relatively low rate during a period of several days. The initial rate of production from carboxyl-labeled 2,4-D is several times that from methylene-labeled 2,4-D. No carbon dioxide is evolved from the ring carbon at position 1.

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FRENCHING SYMPTOMS PRODUCED IN *NICOTIANA TABACUM* AND *NICOTIANA RUSTICA* WITH OPTICAL ISOMERS OF ISOLEUCINE AND LEUCINE AND WITH *BACILLUS CEREUS* TOXIN

ROBERT A. STEINBERG

(WITH TWO FIGURES)

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It has been suggested by the writer that the stages leading to abnormal growth in frenching of field tobacco may be as follows: bacterial soil toxin ⇢ receptor → isoleucine → frenching. The chemical composition of the bacterial soil toxin is unknown as yet. However, natural leucine as well as isoleucine was found to be capable of producing these growth abnormalities in *Nicotiana rustica*. Natural isoleucine can produce the symptoms of frenching in tobacco (**1, 3**) and in *N. rustica* (**5**). The natural isomer of leucine is also effective with *N. rustica* (**5**). The actions of other optical isomers of isoleucine and leucine are unknown.

Frenching of tobacco in the laboratory has been produced with a common soil microörganism, *Bacillus cereus* Fr. and Fr. (**2, 4**). Though this bacillus is not known to invade the tissues of the tobacco plant, rhizosphere and rhizoplane counts increase by 65 and 200%, respectively, when frenching occurs in the field (**6**). Diffusates of *B. cereus* in aseptic culture appear to be capable of exercising a similar action (**4**). The absence of free amino acids in soil and the marked increase of free isoleucine in affected leaves led to the assumption that the bacterial toxin and isoleucine were probably not identical (**7**). No free amino acids could be detected in soil by the Van Slyke method for determining free amino acids which is sensitive to 0.01% or 100 p.p.m., the approximate concentration at which isoleucine would have to be present to be effective.

Data in this paper cover the frenching responses of tobacco treated with optically active isomers of isoleucine, leucine, and two dipeptides of leucine. The effects of various impure preparations of bacterial toxin from *B. cereus* were also studied.

Methods

The tests upon seedlings of the action of organic compounds and of bacterial toxin were made necessarily in aseptic culture. The methods used have been published in previous papers (**1, 2**). Briefly, they consisted in growing tobacco seedlings in aseptic culture on mineral agar from sterilized seed. Either 200 ml. or 1000 ml. flasks were used. A light intensity of about 500 foot-candles was furnished by white fluorescent lamps. Usually, the experiments with *N. tabacum* were carried out in 200 ml. flasks at 25° C, and those with *N. rustica* in 1000 ml. flasks at room temperature. The

former were grown for four to six weeks, the latter, two to three months. All tests were in duplicate.

Two tobacco varieties were used in these experiments. One was Maryland Medium Broadleaf, the other was Havana 142, a Wisconsin cigar tobacco. The *N. rustica* variety was Mahorka 1 originating in Siberia (**5**).

D-Leucine, L-alloisoleucine, and D-alloisoleucine, were obtained through the courtesy of Dr. Jesse P. Greenstein of the U. S. Public Health Service; and γ-aminobutyric acid from Dr. F. C. Steward of Cornell University. The other amino acids were commercial preparations.

Results

Optical isomers

The data for *N. rustica* in table I were obtained with one liter flasks at room temperatures for a growth period of several months. It will be noted that only the natural isomers of isoleucine and leucine belonging to the l-family caused the seedlings to french. Leucine appeared to be more effective than isoleucine. Far more effective than either of these was L-alloiso-

TABLE I

EFFECTS OF THE OPTICAL ISOMERS OF ISOLEUCINE AND LEUCINE ON GROWTH OF *N. rustica* (MAHORKA 1).

Compound	p.p.m.	At budding			At flowering			Symptoms of toxicity
		Days	Leaves		Days	Leaves	Bracts	
				Cm.				
None	....	30	9.3	13.0	36.3	9	5.0	Normal
L(+)-Isoleucine	100	....	....	13.8*	....	....	....	Chlorotic, narrow leaves
	200	....	....	10.3*	....	....	....	Strap leaves
	300	....	....	10.0*	....	....	....	Strap leaves
D(−)-Isoleucine	100	29	9.5	11.8	35.5	8.5	5.0	Normal
	200	37.5	8.5	8.5	45.0	10.5	3.0	Small
DL-Isoleucine	100	82.5	15.0	15.0	82.0			Chlorotic, narrow leaves
	200	....	....	11.8*	....	....	....	Strap leaves
L(−)-Leucine	50	38.0	10.0	8.0	46.0	11.0	3.5	Chlorotic, narrow leaves
	100	51.5	15.8	8.3	60.8	17.5	2.5	Strap leaves
	200	45.0	19.0	10.0	53.0	22.0	0	Strap leaves
D(+)-Leucine	50	....	....	6.0*	....	....	....	Interveinal chlorosis
	50	94.0	15.0	6.0	102.0	15	0	Marginal chlorosis
	100	....	....	3.8*	....	....	....	Marginal chlorosis
L-Alloisoleucine	50	....	....	1.0*	....	....	....	Strap leaves
	100	....	....	1.0*	....	....	....	Strap leaves
	200	....	....	1.0*	....	....	....	Strap leaves
D-Alloisoleucine	200	40.0	8.5	6.0	47.5	9.0	5.0	Normal
L-Leucylglycine	200	45.5	10.5	13.5	47.5	11.5	3.0	Normal
Glycyl-L-Leucine	200	34.0	8.0	8.5	42.0	10.0	3.0	Normal
γ-Aminobutyric acid	50	33.0	8.0	11.5	38.0	8.0	5.0	Normal
	100	33.0	8.0	10.5	38.0	8.0	5.0	Normal

*Height at harvest.

TABLE II

EFFECTS AT 200 P.P.M. OF OPTICAL ISOMERS OF ISOLEUCINE AND LEUCINE ON GROWTH OF MARYLAND MEDIUM BROADLEAF TOBACCO.

Compound	Symptoms of toxicity	
	Time in days	Description
None	....	Normal
L-Alloisoleucine	6	First leaf formed was strapped
D-Alloisoleucine	....	Normal
D-Leucine	7	Retarded; pale; small round leaves, long petioles
L-Leucine	....	Normal
L-Isoleucine	13	Strap leaves
D-Isoleucine	....	Normal

leucine. D-Leucine, D-isoleucine, and D-alloisoleucine did not cause frenching responses to appear. Coupling of L-leucine with glycine to form a dipeptide destroyed its frenching properties. The tests with γ-aminobutyric acid are included because its action on growth of *N. rustica* seedlings had not been studied previously.

The action of the optical isomers of isoleucine and leucine on growth of Maryland Medium Broadleaf tobacco is illustrated in table II. Only L-isoleucine and L-alloisoleucine were effective in causing frenching symptoms. L-Alloisoleucine was more effective in causing growth abnormalities than was L-isoleucine. A growth period of four weeks at 25° C, was used in this experiment. The flasks were of 200 ml. capacity.

Frenching of tobacco ordinarily starts with a reticular chlorosis of the unexpanded upper leaves, and an inhibition in elongation of the main axis. If severe enough, the new leaves that develop are strapped, *i.e.*, consist almost entirely of midribs. A notation of strap-leaves indicates usually that the reticular chlorosis stage had occurred also. Where strap-leaves were formed after a very brief interval, the reticular chlorosis stage may be bypassed. The action of L-alloisoleucine in table II at 200 p.p.m. took place in such a manner.

A comparison of the relative effectiveness of isoleucine and alloisoleucine was made at 25° C with Maryland tobacco seedlings. Flasks of 200 ml.

TABLE III

RELATIVE EFFECTIVENESS OF L-ISOLEUCINE AND L-ALLOISOLEUCINE IN CAUSING THE FORMATION OF FRENCHING SYMPTOMS IN MARYLAND MEDIUM BROADLEAF TOBACCO.

Compound	Reticular chlorosis		Narrow leaf		Strap-leaves	
	Days	p.p.m.	Days	p.p.m.	Days	p.p.m.
L-Isoleucine	16	8	16	16	22	40
L-Alloisoleucine	19	2	12	4	12	8

capacity were used. It is evident on examination of table III that whether comparison is made at the reticular chlorosis stage, at formation of narrow leaves, or at strap-leaves, the results are the same. L-Alloisoleucine was about four times more effective than L-isoleucine in causing the formation of symptoms of frenching in tobacco seedlings.

Figure 1 illustrates the relative effectiveness of these two isomers at the 16 p.p.m. level. The seedlings were 41 days old. Addition of L-alloisoleucine resulted in greatly decreased growth of seedling which showed four strap-leaves. The seedling treated with L-isoleucine at 16 p.p.m. was much larger, and its leaves were perceptibly narrow but not strapped.

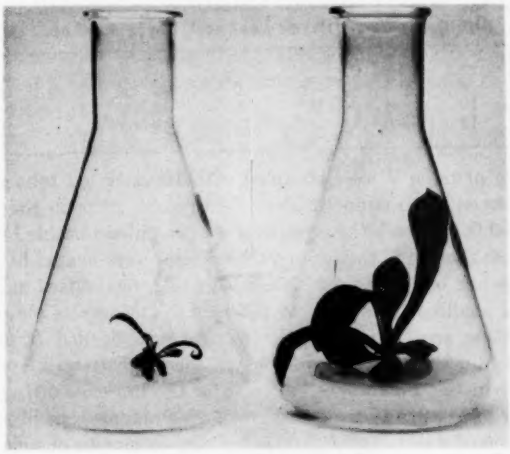

FIG. 1. Maryland Medium Broadleaf seedlings 41 days old Seedling at left treated with L-alloisoleucine and that on right with L-isoleucine. Both amino acids were used at the 16 p.p.m. level.

B. cereus TOXIN

Table IV is a repetition and extension of trials previously reported (**4**) for Maryland tobacco seedlings. With none of the methods of inoculation of the agar was there any evidence of bacterial spread. Bacterial growth was localized at the point of inoculation. The cup type of inoculation refers to the use of small sintered-glass Pyrex crucibles capable of retaining bacteria. Only the medium inside the crucible was inoculated. Nevertheless, the products of diffusion from the bacteria caused frenching. Transfers of the agar medium at a distance from the point of inoculation to Bactopeptone broth did not show the presence of viable bacteria. The experiments were carried out at 25° C for four weeks using 200 ml. flasks. Havana 142 is more sensitive to the action of isoleucine than is Maryland Medium Broadleaf tobacco.

TABLE IV

EFFECT OF TYPE OF AGAR INOCULATION (*B. cereus*) ON THE PRODUCTION OF FRENCHING SYMPTOMS IN ASEPTICALLY CULTURED TOBACCO SEEDLINGS.

Inoculation	Maryland Medium Broadleaf				Havana 142			
	Initial chlorosis		Initial strapping		Initial chlorosis		Initial strapping	
	Days	Leaf no.	Days	Leaf no.	Days	Leaf no.	Days	Leaf no.
Control	....	....	....	....	....	....	....	....
Stab	9	4	15	6	12	2	16	5
Smear	10	4	20	6	12	2	16	5
Cup	13	6	....	....	12	....	28	7

The data of table V were obtained with Havana 142 tobacco grown in 200 ml. flasks at room temperatures. The results given in the table under *B. cereus* viable are much the same as those presented in table IV under Havana 142. At the end of these tests, the cultures were heated to 100° C, and the seedlings and cups removed. They were then resterilized at 120° C, and fresh sterile seedlings inserted into the agar. The results obtained on the second planting are indicated under the columns headed *B. cereus* dead. Frenching had occurred in the presence of the dead bacteria and its heated diffusion products. Bacteriological tests for the presence of living bacteria in the resterilized cultures were negative with Bactopeptone broth.

The action of a sterilized *B. cereus* culture on growth of tobacco is illustrated in figure 2. Chlorosis and extreme strapping of the leaves are evident. The appearance of the seedling is quite characteristic of frenching.

Discussion

The morphological abnormalities characteristic of frenching are: (a) the cessation of stem and branch growth, flowering also being inhibited or prevented; (b) reticular chlorosis of incompletely developed top leaves in the early stages of the disease; and (c) non-expansion of leaf lamina, *i.e.*, strap-

TABLE V

EFFECT OF HEAT ON THE CAPACITY OF *B. cereus* CULTURES TO CAUSE FRENCHING SYMPTOMS IN HAVANA 142 TOBACCO.

Inoculation	*B. cereus* viable				*B. cereus* dead			
	Initial chlorosis		Strap leaves		Initial chlorosis		Strap leaves	
	Days	Leaf no.	Days	Leaf no.	Days	Leaf no.	Days	Leaf no.
Control	....	....	....	....	....	....	....	....
Stab	8	3	....	....	9	2	....	....
Smear	....	....	....	....	....	....	....	....
1 cup	7	3	22	7	9	2	13	3
2 cups	6	2	20	4	13	....	24	....

ping of immature leaves. These three hormone-like effects were limited to L-isomers of alloisoleucine, isoleucine, and leucine. Substitution in the amino or carboxyl groups of L-leucine destroyed its frenching activity. It is possible, therefore, that neither leucine nor isoleucine exercises a direct action in causing frenching symptoms, since leucine is ineffective with tobacco and more effective than L-isoleucine with *N. rustica*. Both possibly represent intermediate stages in causation of this response.

Whether L-alloisoleucine enters into these reactions is unknown. It is not known to occur naturally. Were it not for this, the simplest explanation with the facts on hand would be that L-alloisoleucine is the bacterial toxin causing frenching. Its effective minimal range for chlorosis and strapping

FIG. 2. Maryland Medium Broadleaf seedling grown in a heat-sterilized culture of *B. cereus* for 49 days and showing symptoms of frenching.

in agar cultures is 2 to 8 p.p.m. or less. In water culture, it should prove 5 to 10 times more effective, if it performs similarly to isoleucine. No definite conclusion can be reached, however, until the chemical structure of the bacterial toxin becomes known.

Summary

Nicotiana tabacum (Maryland Medium Broadleaf and Havana 142) and *N. rustica* (Mahorka 1) were grown aseptically on mineral agar under about 500 fc of white fluorescent light. Addition of either L-isoleucine or L-alloisoleucine produced symptoms of frenching in both species. L-Leucine was also effective with *N. rustica*. Relative effectiveness on tobacco was alloisoleucine > isoleucine; on *N. rustica*, alloisoleucine > leucine > isoleucine. L-Alloisoleucine was four times more effective (minimal range 2 to

8 p.p.m.) than L-isoleucine with tobacco. D-Isoleucine, D-leucine, and D-alloisoleucine did not cause frenching symptoms. Neither did glycyl-L-leucine nor L-leucyl-glycine. A heat stable toxin from *Bacillus cereus* Fr. and Fr. also caused production of frenching symptoms in tobacco.

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VARIETAL ROOT AND TOP REGENERATION OF FIG CUTTINGS AS INFLUENCED BY THE APPLICATION OF INDOLEBUTYRIC ACID

JULIAN C. CRANE AND TARA S. MALLAH

(WITH SIX FIGURES)

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Introduction

The practice of applying substances to cuttings before planting them has been confined largely, in recent years, to the use of certain synthetic compounds valuable for their property of initiating root growth. The discovery of indoleacetic acid in plants, a compound capable of laboratory synthesis, initiated numerous investigations with regard to its use as an agent for promoting rooting in cuttings. In 1935, COOPER (**2**) found this substance to stimulate greatly the formation of roots on cuttings of various species. Many synthetic growth substances have since been found to aid the rooting of cuttings (**10**), but the two most commonly used now are indolebutyric and naphthaleneacetic acids.

The four commercial varieties of figs produced in California (Adriatic, Calimyrna, Kadota, and Mission) are all readily propagated from cuttings taken during the dormant season. In the literature dealing directly with the use of growth regulators in fig propagation, very little sound information is available, probably due to the fact that little or no difficulty is ever experienced in propagating figs by cuttings. Although the treatment of fig cuttings with hormones has been shown to be somewhat beneficial (**2, 4, 9**), no experiments which deal with the effect of hormone concentration on root initiation and subsequent development of top and roots have been reported. This investigation was undertaken, therefore, to determine the relative rate and degree of root and top regeneration on cuttings of four varieties of figs as well as their response to various concentrations of indolebutyric acid.

Materials and methods

On December 21, 1949, approximately 100 branches of uniform diameter of each variety, consisting of one-, two, and three-year old wood, were collected from commercial Adriatic, Calimyrna, Kadota, and Mission orchards in Merced County, California. Although the orchards from which the material was collected differed in age, all trees were estimated to be between 20 and 30 years of age. From the most uniform 80 branches of those collected of each variety, cuttings were made which consisted of one-year old wood with its terminal bud and sufficient two-year old wood to make each cutting eight inches in length. One- and two-year old wood averaged five and three inches in length respectively. Just previous to placing the cuttings in the

indolebutyric acid (IBA) solutions, fresh cuts were made through their basal nodes. As soon as the basal cuts were made, 20 cuttings of each variety were placed in gallon cans containing a water solution of 100, 150 and 200 p.p.m. of IBA. Enough of the hormone solution was contained in each can to immerse the basal inch of the cuttings. Untreated control cuttings were placed in cans containing a similar quantity of water only. All cuttings were allowed to remain in these solutions for a period of 22 hours.

IBA was used in this investigation because the general consensus of opinion is that this compound is the best one for stimulating root initiation. It appears to be more effective than indoleacetic acid for a large number of species and, in addition, has a wider range of effectiveness without causing injury to cuttings than naphthaleneacetic acid. Each cutting was labeled according to variety and the hormone treatment that it received. The cuttings were placed in a propagating bed in the greenhouse on December 25, having been held in moist sawdust from the time they were prepared until they were placed in the hormone solutions. Each cutting was planted so that its terminal bud was just above the surface of the rooting medium. The propagating bed was filled to a depth of 12 inches with a mixture of three parts by volume of sand to one part of peat moss. Bottom heat was provided by electric resistance cables and a temperature of 70 degrees F was maintained. The propagating bed was divided into 20 equal portions or blocks, and in each block a single cutting of each of the 16 treatments was planted at random. Five blocks of cuttings were removed at random from the propagating bed on January 30, 1950, approximately one month after planting, and five blocks were subsequently removed on February 18, March 11, and April 7. Upon removal the roots were carefully washed free of sand and peat moss, and the five cuttings of each respective treatment were grouped.

On each sampling date, data were collected with regard to root distribution on the cuttings; condition of the underground portion as regards swelling, bark splitting, or other injury; number, fresh weight, and total linear length of roots per cutting; fresh weight of top and the apparent injury, if any. The degree of injury as a result of IBA treatment was classified as minor injury, which consisted of swelling, bark splitting, and a sparsity of roots on the basal-most portions of the cuttings, moderate injury, characterized by the absence of roots due to their deterioration on the basal portions of the cuttings, and severe injury, in which deterioration of the roots and their supporting tissues had taken place on the basal portions of the cuttings.

The untreated controls were used in determining the relative rate and degree of root and top regeneration in the different varieties of cuttings. In ranking the varieties in respect to rate and degree of regeneration, each of the four measurements made on the material (number of roots, fresh weight of roots, length of roots, and fresh weight of top) were taken into consider-

ation and given equal value. Since the data for fresh weight and length of roots showed the same trends as the data on number of roots, only the latter, together with data on fresh weight of tops, are presented here.

Results

The data presented graphically in figure 1 indicate the relative degree of root and top regeneration on cuttings of the four varieties. The average number of roots and the weight of top of the Adriatic cuttings were at least twice the values for any other variety. The number of roots and the weight of top of Calimyrna cuttings were less than one half of the values for Mis-

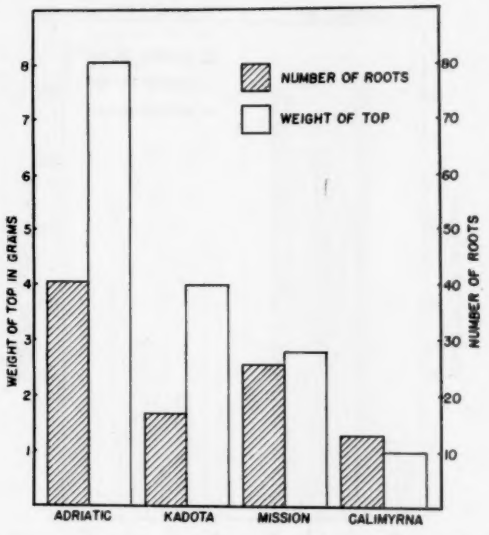

FIG. 1. Average number of roots and weight of top for all samplings of untreated control cuttings.

sion cuttings and from one third to one eighth of the values for Adriatic cuttings. The Kadota and Mission varieties were about equal in regard to degree of root and top regeneration; but considering weight and length of roots, the Kadota was somewhat better than the Mission.

The cuttings of the Adriatic variety initiated roots and tops considerably more rapidly than did those of the other varieties. On the first date of sampling, the Adriatic cutting had about three times as many roots as did any of the others. The Calimyrna variety was the slowest in the rate of regeneration; most of the roots and tops were made during the interval between the third and last samplings. The Kadota and Mission varieties occupied similar intermediate positions as regards rate of regeneration. Based

upon the whole of the control data, the Adriatic had the greatest rate and degree of root and top regeneration followed in decreasing order by the Kadota, Mission, and Calimyrna varieties.

An examination of figures 2, 3, 4, and 5 shows that treatment with IBA of cuttings of all varieties except the Calimyrna resulted in injury in varying degree. Cuttings of the Calimyrna variety exhibited no symptoms of injury at any of the concentrations of IBA used.

Injury, which increased in severity with increase in IBA concentration, was manifest in the early stages by a swelling and splitting of the bark on the basal portion of the cuttings. Although numerous roots were produced

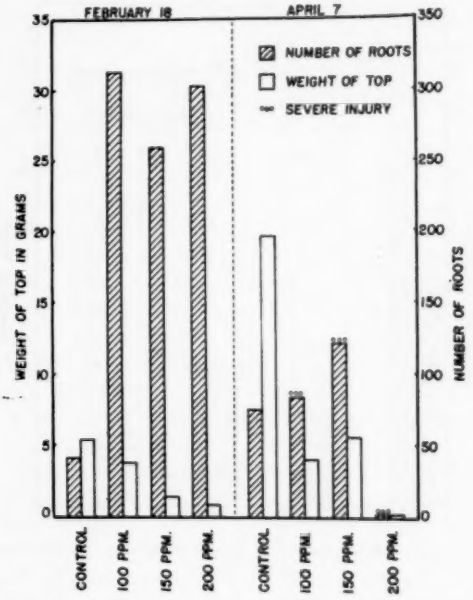

FIG. 2. Effect of IBA concentration on the average number of roots and fresh weight of top per Adriatic cutting sampled on February 18 and April 7. Cuttings treated with 200 p.p.m. of IBA were dead when sampled on April 7.

at this location, they gradually deteriorated along with the stem tissue from which they were initiated. In only one case, that of Adriatic cuttings treated with 200 p.p.m. of IBA, was injury so severe as to cause death of the cuttings. Injury appeared earliest and was most severe in the Adriatic cuttings and occurred in decreasing order in the cuttings of Kadota, Mission, and Calimyrna varieties. It would seem significant that the order in which the varieties are listed with respect to the degree of injury as a result of application of identical concentrations of IBA is exactly the same as the order of listing of the untreated control cuttings with regard to the rate and de-

gree of root and top regeneration. It is evident that the greater the natural potentialities a variety of cutting has for regeneration, the greater is the degree of injury accompanying the application of a root-promoting substance such as IBA. The difference in root distribution between the cuttings treated with IBA and control cuttings was quite striking. In contrast to the control cuttings where most of the roots were initiated at or near the basal cut, roots on the treated cuttings were scattered at random over the lower four to five inches (fig. 6).

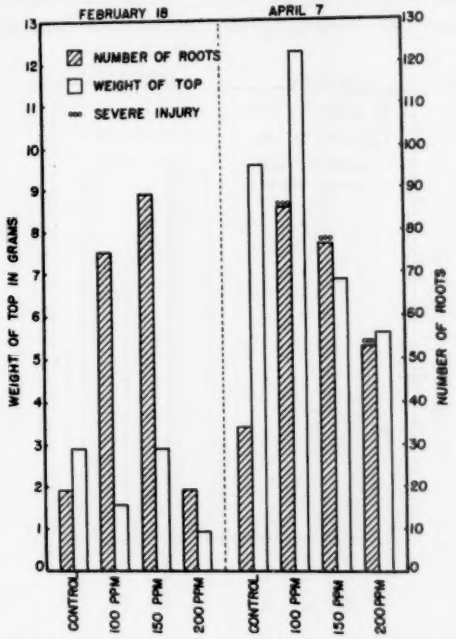

FIG. 3. Effect of IBA concentration on the average number of roots and fresh weight of top per Kadota cutting sampled on February 18 and April 7.

From the standpoint of the percentage of cuttings that survived or those that produced new plants, there was no advantage whatsoever in treating the four varieties with IBA. At last sampling, all control cuttings had rooted and produced tops. Application of IBA was advantageous in the case of the Calimyrna variety only in the rate and degree of root and top regeneration and not in the percentage of survival. Because of the injurious effects accompanying the use of IBA on all varieties of cuttings except the Calimyrna, the results of this investigation may be summarized with the generalized statement that the application of IBA increases the rate and degree of root and top regeneration in the Calimyrna variety only. A more

detailed examination and interpretation of the data, however, indicates an interesting relationship between the response of the four varieties of cuttings to IBA treatment and their potentialities for root and top regeneration.

Data on the average number of roots and weight of top per cutting for only the second and last sampling dates are presented graphically in order that this relationship might be illustrated more clearly. Some cuttings had failed to root on January 30, the first sampling date, and significant comparisons, therefore, could not be made. Some of the cuttings particularly those of the Adriatic variety, on March 11, the third sampling date, were moderately injured as a result of IBA treatment, and some of the roots had

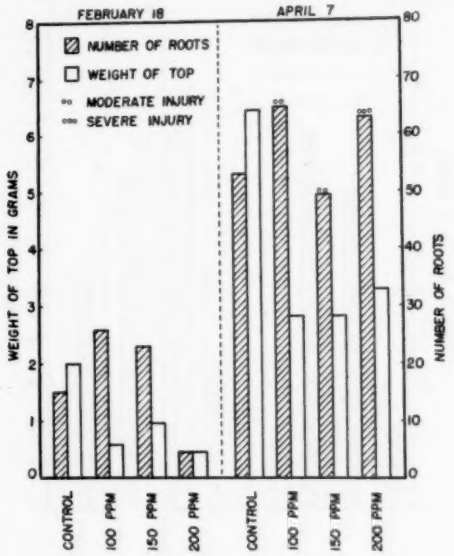

FIG. 4. Effect of IBA concentration on the average number of roots and fresh weight of top per Mission cutting sampled on February 18 and April 7.

deteriorated and separated from the cuttings when they were removed. Since no injury was apparent on any of the cuttings on the second date of sampling, February 18, the data obtained on this date show the maximum rooting response obtained in each case as a result of IBA treatment. Data obtained at the last sampling are presented along with those of the second to show what took place during the interim.

At the second sampling, February 18, the data in figures 2, 3, 4, and 5 indicate that all varieties of cuttings, compared with their respective controls, were stimulated either in number of roots or weight of top or both by the application of a particular concentration of IBA. The most striking response was obtained with Adriatic cuttings that had been treated with 100

p.p.m. of IBA (fig. 2). The treated cutting had almost eight times as many roots as the control cutting. Inhibition to top growth progressively increased, however, as the concentration of IBA increased until at 200 p.p.m. of IBA, top growth was about one fifth that of the controls. When sampled on April 7, cuttings previously treated with 200 p.p.m. of IBA were dead while those treated with 100 or 150 p.p.m. were severely injured. Top growth on the treated cuttings was one fourth to one fifth as much as on control cuttings.

Kadota cuttings at second sampling were found to have been stimulated, as far as root production is concerned, by concentrations of 100 and 150

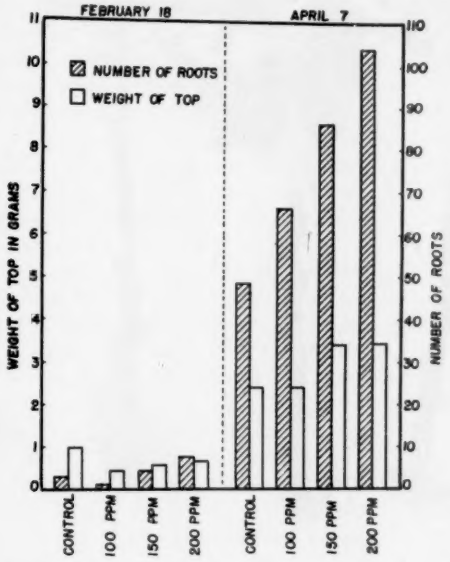

FIG. 5. Effect of IBA concentration on the average number of roots and fresh weight of top per Calimyrna cutting sampled on February 18 and April 7.

p.p.m. of IBA (fig. 3). Top growth, however, at these concentrations, was less or only equal to that on the control cuttings. Root production on cuttings treated with 200 p.p.m. of IBA was about equal to that on the controls and top growth was inhibited. At last sampling, all treated cuttings were severely injured (fig. 3). The amount of root and top growth per cutting was found to be inversely related to the concentration of IBA.

The response of Mission cuttings to IBA treatment was similar to that of Kadota cuttings except that injury was somewhat less. At last sampling, although the number of roots per cutting in the majority of the IBA treatments was greater than on the controls, top growth was greatly inhibited (fig. 4). Injury, as a result of hormone treatment, varied from moderate at

the 100 and 150 p.p.m. levels to severe at the 200 p.p.m. concentration. In contrast to the effects on cuttings of Adriatic, Kadota and Mission varieties, data in figure 5 clearly indicate that with a progressive increase in concentration of IBA there was a progressive increase in average number of roots on Calimyrna cuttings. Although the concentration of IBA which will induce maximum rooting in Calimyrna cuttings without injury has not been determined in this experiment, the data indicate that cuttings of the type

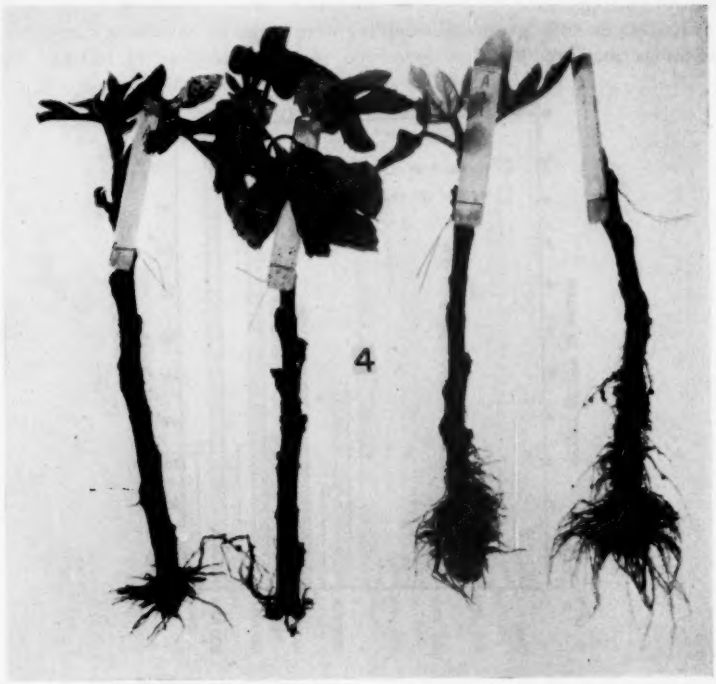

FIG. 6. Untreated Adriatic cuttings (left) and those treated with 200 p.p.m. of IBA (right). Second sampling, February 18, 1950.

described here treated with IBA at concentrations of 100 p.p.m. to 200 p.p.m. may be expected to regenerate roots and tops more rapidly and to a greater extent than untreated cuttings.

Discussion

Cuttings taken from closely related varieties of Hibiscus have different capacities for initiation of roots (5). Since White hibiscus cuttings rooted readily only when auxin was applied in the presence of cofactors supplied by the leaves on the cuttings, GREGORY and VAN OVERBEEK (5) suggested that cuttings of the White variety are difficult to root because they are de-

ficient both in auxin and cofactors supplied by the leaves. The cofactors were later identified to be carbohydrate and nitrogenous substances (**8**). Since there was a great increase in number of roots produced per fig cutting as a result of IBA application, it is evident that carbohydrate and nitrogenous substances were not limiting. Apparently auxin was the limiting factor since in all varieties maximum root initiation was stimulated by a particular concentration of IBA. Thus the natural auxin content in each variety of cutting was apparently lower than that which was necessary to bring about the initiation of the maximum number of roots per cutting.

Injury, as a result of IBA treatment, appeared first and was most severe in Adriatic cuttings, and occurred in decreasing order in cuttings of the Kadota, Mission, and Calimyrna, there being no injury in the latter. It will be noted that the order in which the varieties are listed with respect to degree of injury is identical to the order of listing regarding the rate and degree of root and top regeneration in the untreated controls. The greater the natural potentialities present for regeneration in a particular variety of cutting, the more severe was the degree of injury accompanying the external application of IBA. The significance of the fact that the varieties initiate growth in the spring in exactly this same order is not clearly understood. The idea suggests itself, however, that breaking of dormancy in plants may be closely associated with auxin level.

The differential response of the various varieties of cuttings to applications of IBA illustrates the regulatory influence of auxin upon root and top regeneration in fig cuttings. Cuttings of the Adriatic, Kadota, and Mission varieties were greatly stimulated in root production at a certain concentration of IBA, but top growth was inhibited. Generally, as root production was increased as a result of an increase in concentration of IBA, top growth decreased. STUART (**7**) found a similar response in kidney bean cuttings to applications of indoleacetic acid. He attributed the progressive suppression of top growth with progressive increase in concentration of indoleacetic acid to the movement of carbohydrates and nitrogen away from the apical regions as these materials were mobilized in the region of indoleacetic acid application.

Since cuttings of the Adriatic, Kadota, and Mission varieties were either killed or severely injured by a 200 p.p.m. concentration of IBA, a concentration that greatly stimulated root and top regeneration on Calimyrna cuttings without injury, the data indicate that the Calimyrna variey is lower in natural auxin content or is less susceptible to IBA toxicity. The different pollination requirements of the varieties in question suggest that the former is the factor responsible for the differential response of cuttings of these varieties to identical concentrations of IBA. The Adriatic, Kadota, and Mission varieties all set their fruits parthenocarpically, while cross-pollination and fertilization are essential for fruit setting in the Calimyrna variety, unless a hormone is applied externally (**1, 3**). The same relationship of auxin content to fruit setting in the fig appears to exist, as has been reported by GUSTAFSON (**6**) for oranges, lemons, and grapes in which the auxin con-

tent in ovaries of the varieties that produce fruits parthenocarpically is higher than in the ovaries of varieties which do not.

The technique used here and the results obtained indicate the importance of the time factor in experiments of this type. A large portion of the data in the literature on the response of cuttings to hormone treatment represents the condition of the treated cuttings at one observation only, in most cases, three or four weeks after treatment. Had observations and recommendations been made only at the second sampling in this investigation, it is evident how erroneous the conclusions would have been in regard to treatment of fig cuttings.

Summary

Tip cuttings of the four fig varieties produced in California were rooted in a propagating bed supplied with bottom heat in a greenhouse. Untreated Adriatic cuttings were found to have the greatest rate and degree of root and top regeneration, followed in decreasing order by the Kadota, Mission, and Calimyrna varieties.

Treatment with indolebutyric acid (IBA) at concentrations of 100, 150, and 200 p.p.m. resulted in injury to cuttings of all varieties to varying degree except in the case of the Calimyrna. Cuttings of this variety exhibited no symptoms of injury at any of the concentrations used. Injury appeared earliest and was most severe in the cuttings of the Adriatic variety and occurred in decreasing order in the cuttings of the Kadota, Mission, and Calimyrna varieties, there being none in the last. The order in which the varieties are listed with respect to the degree of injury as a result of identical concentrations of IBA is exactly the same as the order of listing of the untreated cuttings with regard to the rate and degree of root and top regeneration. The greater the natural potentialities a variety of cutting has for regeneration, the greater is the degree of injury accompanying the application of the root-promoting substance.

From the standpoint of the percentage of cuttings that produced new plants, there was no advantage in treating the four varieties with IBA, since all untreated cuttings produced roots and tops. Applications of IBA, in the case of the Calimyrna variety increased the rate and degree at which root and top regeneration took place.

The Dow Chemical Company supplied the indolebutyric acid used in this study.

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MEASUREMENT OF METABOLIC GAS EXCHANGE WITH A RECORDING MASS SPECTROMETER

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(WITH FIVE FIGURES)

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Introduction

This paper describes a new method for measuring the gaseous metabolism of cells, tissues, and whole organisms. The method incorporates certain features which offer unique advantages for physiological studies. In principle, measurements consist of successive analyses, at frequent intervals, of the partial pressures of several constituents of the gas phase over the biological material under observation. A mass spectrometer is employed as the measuring instrument.

A mass spectrometer is essentially a special kind of vacuum tube through which the gas or vapor being analyzed is flushed continuously at a low pressure. A hot tungsten filament generates an electron beam at one end of the tube (the source), and positive ions of the gas are created there by electron bombardment. These ions are accelerated in an electric field and focused into a beam which projects down the tube. The ion beam is resolved into its mass components by the analyzer, a magnetic field, located part way down the tube. As a charged particle enters the magnetic field, it is deflected by an amount which depends upon the strength of the field and on the velocity mass and charge of the particle. Lighter particles enter the field with greater velocity and hence suffer greater deflection. Thus an ion beam, originating from a mixture of gases having different molecular weights, is resolved into a series of discrete beams according to the respective masses of the constituent ions. Proper adjustment of the ion accelerating voltage and of the field strength of the magnetic analyzer will cause any one of the separate ion beams to come to focus on the collector at the far end of the mass spectrometer tube. The ion current, measured with a vacuum tube amplifier, is proportional to the rate at which ions of the particular mass are formed at the source—proportional, therefore, to the partial pressure of the corresponding component of the gas mixture being flushed through the tube. For general information on the principles of operation of a mass spectrometer and on further technical details, the reader may refer to any of several reviews (**3, 6, 8, 9, 12, 13**).

In our application of the mass spectrometer for analysis of metabolic gases, we operate the instrument at a fixed magnetic analyzer field strength

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and tune in on the desired ion beams by regulating the accelerating voltage. Since we want to effect essentially continuous monitoring of the gas mixture, the accelerating voltage is automatically varied in cyclic fashion tuning to each of several masses in succession. The amplifier output is continuously recorded. Ordinarily no adjustments are necessary once the instrument is set up for the particular analytical sequence desired; the data for the entire experiment are thus collected and recorded completely automatically.

This method has considerable versatility and an exceptionally high degree of analytical specificity because the gaseous constituents are distinguished on the basis of molecular weight which avoids ambiguity in all but a few instances. Since a mass spectrometer distinguishes not only between chemically different gases but also between different isotopes of the same gas, the method is especially applicable to various kinds of tracer experiments.

The use of a mass spectrometer for continuous analysis of a process gas was first described by NIER *et al.* (**10**). Recently ROBINSON *et al.* (**11**) described a similar commercial instrument designed for monitoring continuous industrial processes. KYDD and HITCHCOCK (**4**) apparently were the first to apply these principles to continuous gas analyses in metabolic studies with an instrument described by HUNTER *et al.* (**2**). MILLER *et al.* (**5**) described a portable mass spectrometer designed for respiratory studies on mammals and for use in operating rooms as an aid to the anesthetist.

Apparatus and methods

The apparatus consists principally of a mass spectrometer similar to that described by NIER (**7**). The first instrument used for the kind of studies described herein was constructed in the Physics Department and was modified to facilitate our particular experiments. Provision was made for connecting the gas inlet of the mass spectrometer to one of several types of experimental chambers containing the living material under investigation. Gas from the experimental chamber can diffuse into the mass spectrometer tube at a very slow rate (a few ml. per day) depending upon the size of leak employed. It is this gas which is flushed through the tube at the desired pressure (ca. 10^{-6} mm. Hg). With such a low rate of diffusion through the leak, the gas in the experimental chamber is not seriously depleted during the time of an experiment.

We have used one basic leak design which is of the viscous flow type. The performance of viscous flow leaks was studied by HALSTED and NIER (**1**). The leak was made from copper tubing drawn down to a capillary with an internal diameter of 0.007 inch and pinched by screwing a small steel bar against a backing plate so as to clamp down on the copper capillary soldered to the plate. The leak was cautiously pinched to the desired size which was determined empirically.

The accelerating voltage of the mass spectrometer was controlled by a voltage divider. As part of the divider, a motor-driven potentiometer was arranged to scan a voltage range automatically and to cycle repeatedly over

the preset range thus scanning through the tuned settings for several predetermined masses. A series of intermittent analyses of each of a number of gases was obtained. The voltage output of the mass spectrometer is fed into a recording potentiometer whereupon the data are recorded on a strip chart for subsequent study.

The only changes necessary to adapt a mass spectrometer, designed for routine isotopic analyses of gas samples, to our type of measurements are: (a) provision of the additional leak communicating with the experimental chamber, (b) provision of the voltage divider and automatic tuning, and (c) provision for recording the amplifier output with automatic switching of amplifier sensitivity so that each analysis is recorded at an appropriate sensitivity.

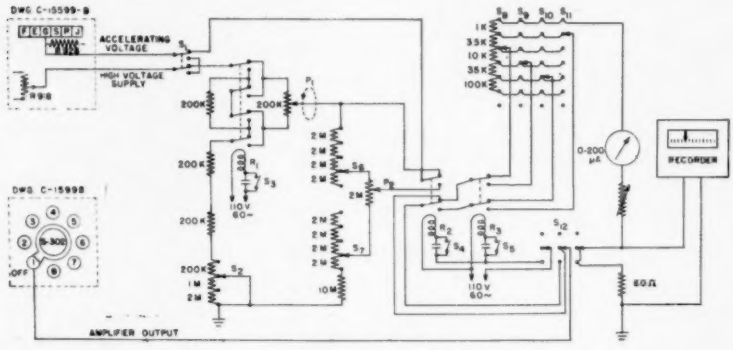

FIG. 1. Accessory circuits for controlling mass spectrometer accelerating voltage and recorder sensitivity. Schematic diagram shows connections to the spectrometer high voltage supply, accelerating voltage control, and amplifier output. Also reproduced within dashed lines are portions of the circuit drawings of the Consolidated Engineering Corp. Mass Spectrometer, Model 21–201 indicating where connections were made in this instrument. See text for description of circuit operation.

These relatively simple changes were incorporated into two different instruments. They do not prevent the use of the spectrometer for the usual isotopic analysis of gas samples according to conventional practice. Pertinent circuit modifications are shown in figure 1. The added circuit components are entirely external to the mass spectrometer chassis with only three leads connecting to the instrument as indicated in the figure. One of the mass spectrometers we have so adapted was a commercial instrument (Consolidated Engineering Corporation Isotope Ratio Mass Spectrometer, Model 21–201). This model instrument is available at a number of institutions where biological problems are under investigation. Figure 1 also indicates the specific points in the circuit drawings of the commercial spectrometer at which connections were made to the accessory circuits. The operation of these circuits may be considered briefly.

Accessory electrical circuits

Referring to figure 1, switch, S_1, can disconnect the voltage divider thus restoring the accelerating voltage control of the mass spectrometer to the usual manual adjustment on the instrument panel. Similarly, setting the panel voltmeter sensitivity control at other than the off position eliminates the added recording circuit. The instrument circuits thus are easily restored to their usual operating condition for isotopic analyses of gas samples introduced into the mass spectrometer manifold. In the opposite position, switch, S_1, introduces a bank of resistors (800K to 4M, depending on the setting of switch S_2) between the spectrometer high voltage and ground. The accelerating voltage of the instrument is picked off from the center tap of the 200K General Radio potentiometer, P_1, which constitutes the top member of the series of resistors. This potentiometer (with end stop removed) is connected through a gear box (with easily changeable gears) to a 2 r.p.m. type K Bodine motor. As the potentiometer is turned by the motor through one revolution, the mass spectrometer accelerating voltage sweeps through a definite fraction of its maximal range. With the sizes of resistors shown, this is 5%, 10%, 20%, or 25% of the range according to the setting of S_2. By changing gears the potentiometer can be turned at several speeds from one fifth to 1 r.p.m. which is about as fast as it is practical to repeat analyses in our experiments.

Switch S_3 controls relay, R_1, which can interchange the scanning potentiometer with the next resistor element thus providing voltage scanning at a lower maximal voltage but over the same voltage range as before. Microswitches S_4 and S_5 are operated by cams attached to the extended shaft of the scanning potentiometer. At a given position in the scanning cycle S_4 operates the relay R_2. As R_2 closes, the mass spectrometer accelerating voltage jumps to a new value determined by the settings of switches S_6 and S_7 and of potentiometer P_2 across their center taps. The other half of the relay R_2 and both sides of relay R_3 are so connected that the amplifier output from the mass spectrometer is read on meter, M, through one of four possible resistances according to the positions of the relays and the setting of switches S_8, S_9, S_{10} and S_{11} connected to R_3. This meter circuit is essentially a replica of the one contained within the mass spectrometer proper, and the sensitivity values obtainable with it are the same as those provided by switch S–302 in the case of the Consolidated instrument.

The voltage output is recorded by a Model 153X17V–X–30 Brown Recording Potentiometer connected across a 60 ohm resistor in the meter circuit. Included in the meter circuit is a variable resistor which can be adjusted so that a full scale reading on the meter corresponds to the full scale of the recorder. With switches S_8, S_9, S_{10} and S_{11} properly set and the cams which activate microswitches S_4 and S_5 adjusted appropriately on the shaft of the motor driven potentiometer P_1, the sensitivity scale on which the different mass peaks are recorded changes during the scanning cycle so that both large and small peaks can be recorded on the strip chart at sensitivities which are respectively appropriate.

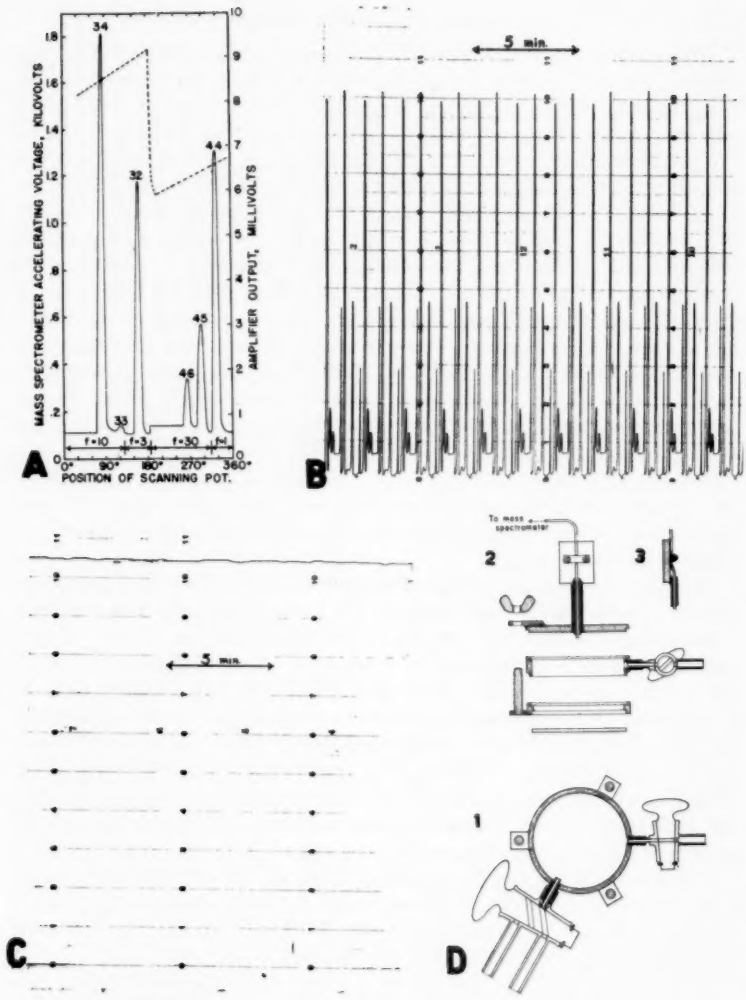

FIG. 2. A. Relation between accelerating voltage (broken line), amplifier output (solid line), and scanning potentiometer position (abscissa) for one complete scanning cycle. Each amplifier output peak is numbered according to the mass represented. Amplification factors (f) show how other peaks are magnified relative to that of mass 44. B. Photograph of sample strip chart data. Same peaks represented as in A. Not to be confused with mass peaks are sharp spikes such as the one, immediately to the left of each mass 32 (highest) peak, which was caused by automatic switching of amplifier sensitivity and other smaller spike, immediately to left of each mass 44 (next to highest)

Operation of mass spectrometer

The dashed line in figure 2 A shows how the mass spectrometer accelerating voltage can be made to vary over two nonadjacent ranges during a scanning cycle. On the same abscissa is reproduced a tracing of part of the strip chart record obtained when the gas being analyzed consisted of a mixture of nitrogen, carbon dioxide, and oxygen which had been enriched in mass 34 ($O^{16}O^{18}$). The apparatus was so adjusted that nitrogen peaks were not recorded. Three isotopic peaks for carbon dioxide and three for oxygen appear in the record. The tops of the peaks are labeled with their respective masses. At the bottom of the graph the amplifier sensitivity factors are indicated. Shifts in sensitivity occur automatically between the 33 and 32 peaks, between the 45 and 44 peaks, and also at the point where the accelerating voltage changes abruptly to a different part of its range. The factors (f) indicate the relative amplification of the spectrometer output voltage for the various mass peaks.

As the cycle repeats, a series of such peak patterns appears on the strip chart constituting a continual succession of analyses as shown in figure 2 B. This figure is a photograph of a section of strip chart upon which is recorded the continual analysis of a gas mixture very similar to the one whose analysis is shown in figure 2 A. Some indication of the reproducibility of our data can be obtained by comparing respective analyses in different cycles over the 20 minutes represented by these data. For certain applications it is not necessary to change amplifier sensitivity or to interrupt the scanning range. To eliminate the operation of relays R_2 and R_3, ganged switches, S_{12}, can be set in the middle position.

In addition to the electrical circuit modifications depicted by figure 1, it was necessary to devise a new mass spectrometer leak since the one incorporated in the commercial instrument is not suitable for our purposes. Otherwise the commercial mass spectrometer was operated in normal fashion and proved to be a dependable instrument for measuring metabolic gas exchange.

The manner in which the data were taken, of course, depended upon the purpose and conditions of the particular experiment. In some instances it was advantageous to record continuously the partial pressure of one particular gas in the experimental chamber in order to observe short term fluctuations. For this purpose the instrument was tuned to the desired mass peak

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peak, which represents the back trace at the end of each scanning cycle. No gas was being evolved or consumed. Cycling period, 1.8 min. C. Photograph of the sample strip chart record of mass 32. Instrument is tuned to oxygen peak and no change in oxygen partial pressure occurs within the apparatus. The fluctuations of 1% indicate the degree of instrument instability. D. Experimental chamber constructed of brass in three sections with circular glass window on bottom. 1. Top view showing insertion of stopcocks into middle chamber section. 2. Exploded view showing all three sections above glass bottom-plate. Mass spectrometer leak assembly attached to top section. Copper capillary leak tubing extends into chamber. 3. Side view of leak assembly only showing copper tubing pinched by clamp to form leak.

and a continuous record was obtained. Figure 2 C is a photograph of such a record. The mass spectrometer was tuned to mass 32 and the gas being analyzed was undergoing no change in oxygen partial pressure. Extreme fluctuations in such records typically are about 1% or less. In most instances, however, our investigations have required analyses of two or more gases or of more than one isotopic species of the same gas. Then, automatic scanning was employed as indicated above to provide a mass peak pattern that was repeated at intervals whose length depended upon the cycling rate.

Experimental vessels

The requirements of the high pressure side of the apparatus, *i.e.*, the experimental chamber, vary with the experiment so that a variety of designs may be needed. Three different designs are illustrated and suffice as examples of what has been found in practice to be useful. Figure 2 D shows a glass-bottom chamber of 38 ml. gas space with inlet and outlet tubes attached. The chamber consists of three sections which fit together on rubber gaskets and are clamped by three bolts and wing nuts. External to the experimental chamber but soldered to the top section is a loop of brass tubing (not shown in the figure) which connects to inlet and outlet pieces of one quarter inch copper tubing. Water may be circulated through this to provide for temperature control since the chamber when in use was not placed in a bath but was surrounded by air.

Into the center of the top section of the chamber a metal capillary tube is inserted through a one quarter inch brass stud. The leak into the mass spectrometer is located at a constriction in this capillary as near as possible to the chamber. The top section thus is attached to the mass spectrometer by this capillary tube which leads directly to the spectrometer gas inlet. The top section is fixed in position by a clamp attached to the frame of the mass spectrometer which holds the stud vertical and supports the whole chamber when closed and in use. A Pyrex plate, three sixteenths of an inch thick, cemented to the bottom section of the chamber provides a circular window through which the contents may be illuminated. For such purposes we have used a horizontal optical bench with light source, lenses, and filters as desired and the light was projected upward into the chamber with a right-angle prism. The middle section of the chamber is provided with two openings to which short pieces of one quarter inch brass tubing are soldered. Capillary bore glass tubing is sealed with wax inside of these brass tubes and glass stopcocks provide for closure of these openings through which the chamber can be evacuated or flushed with gas mixtures as desired. The chamber, of course, must be vacuum tight. In use it is assembled with the biological material (*e.g.*, a plant leaf) resting on the glass bottom-plate; the appropriate gas mixture is introduced; the chamber is closed and observations are begun.

The chamber was suitable for experiments with leaves, leaf sections, or other plant parts. However it could not be used for studying algal or

chloroplast suspensions. When working with suspensions it is of course imperative that the liquid be agitated so that soluble constituents of the gas phase shall remain practically in equilibrium with the liquid. To meet this requirement one must either shake the experimental chamber or bubble gas through the suspension. Both methods have been used.

Figure 3 shows apparatus designed to circulate gas in a closed system in which the experimental vessel is one compartment. This vessel is a flat Pyrex glass cell 35 mm. in diameter and 10 mm. thick. The whole unit was constructed on an apparatus rack and the cell protrudes from the plane of

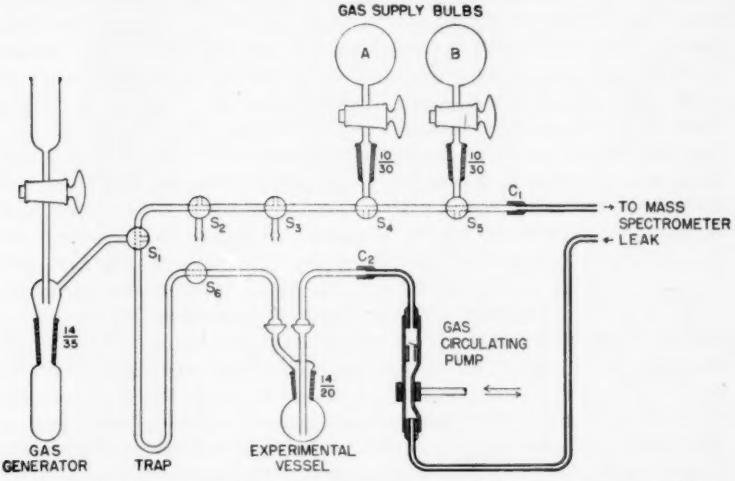

FIG. 3. Gas mixing and circulating system. Stopcocks S_2 and S_3 control flushing of system and addition of background gas. Stopcocks S_4 and S_5 control supplies of special gases from bulbs A and B. Stopcocks S_1 and S_6 control addition of gas from generator. The trap, when immersed in a liquid nitrogen bath, facilitates addition of CO_2 from generator as described in text. Connections C_1 and C_2 are turned brass sleeves waxed to inserted glass tubing and soldered to copper tubes leading to mass spectrometer leak and to gas circulating pump. Experimental vessel is a flat cylinder 1 cm. thick and with 10 ml. useful volume. Contents of vessel are stirred by a bubbling gas stream.

the apparatus so that it may be surrounded by a small water bath whenever required. It is so oriented that the cell contents are easily illuminated horizontally.

PREPARING GAS MIXTURES

Gas may be introduced into the system shown in figure 3 in three ways. Nitrogen or helium, for example, may be flushed through the system in quantity; stopcocks S_2 and S_3 serve as inlet and outlet ports. More precious gases (isotopically enriched), contained in supply bulbs A or B in figure 3, can be introduced by diffusion into a previous evacuated section of the system, for example, between S_3 and S_5. For introducing gases produced from

solid or liquid precursors (radioactive carbon dioxide) the gas generator is used. To produce carbon dioxide from solid carbonate, the generator is evacuated through S_1 and S_2. At the same time the trap also is evacuated to S_6. The trap is surrounded by a liquid nitrogen bath and sulphuric acid is run into the generator from above. The carbon dioxide evolved is frozen out in the trap. The generator is closed off at S_1. The trap is warmed as the pressure is brought to one atmosphere with background gas.

Gas circulating pump

During an experimental run the gas in the system must be circulated to insure representative sampling as well as equilibrium between gas and liquid phases. The pump which accomplishes this must be tight to the outside atmosphere and must pump against a water head of a few centimeters at most. Both rubber tubing and all-metal pumps have been tried. There seems to be no material advantage in employing anything more complicated than the simple type shown schematically in figure 3. This pump was constructed out of short lengths of brass and rubber tubing and was fitted with circular flap valves cut from a sheet of dental dam rubber. The valves were cemented at one point with apiezon wax to the square turned ends of the brass tubing as suggested by the illustration. The internal volume of such a pump can easily be kept small which has been important in some experiments with our apparatus. The intermittent compression of the rubber tubing section between the valves was accomplished by a shaft connected eccentrically to a motor driven flywheel. The motor speed was controlled by a variac and the pump was run at several hundred strokes per minute. Thus operated, the capacities of several pumps of this type were between 100 and 200 ml. per minute. At this rate of bubbling of gas through the experimental vessel, very thorough agitation of the suspension was achieved.

Adaptation to Warburg-type vessel

For studying materials with annoying tendencies to foam (*viz.*, certain algal and chloroplast suspensions) it was found necessary to avoid bubbling, and the experimental vessel illustrated in figure 3 was replaced by a rectangular Warburg-type vessel shown in figure 4 A attached to a joint but not to the conventional Warburg manometer. This joint with gas inlet and outlet tubes was attached to a manometer holder in the same position as is usual for a Warburg vessel with manometer. Thus the experimental vessel can be shaken by a mechanical shaker in a constant temperature Warburg bath of commercial design (J. H. Emerson Co., Cambridge, Massachusetts). A mass spectrometer experiment thus can be run in one vessel with several ordinary Warburg vessels being run simultaneously beside it in the same bath. The gas inlet and outlet tubes of the vessel shown in figure 4 A were connected to the rest of the gas circulating system by metal capillaries and short lengths of rubber tubing so arranged as to insure sufficient flexibility for shaking the experimental vessel.

Mass spectrometer leak

When the apparatus was operated in connection with the modified commercial mass spectrometer it was found convenient to locate the gas leak close to the spectrometer tube; and a new leak assembly (fig. 4 B) was bolted to the aluminum plate upon which the glass manifold of the instrument is mounted. From valve V_2 of this assembly, copper tubing communi-

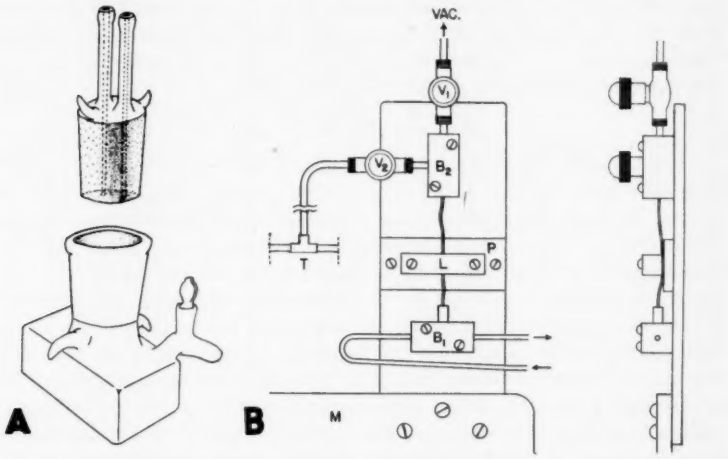

Fig. 4. A. Warburg-type vessel. The vessel is supported by a 24/25 standard taper joint into the male part of which are incorporated gas inlet and outlet tubes. These tubes are connected by short pieces of rubber pressure hose to metal tubing leading to the gas circulating system. It is connected in place of the experimental vessel shown in figure 3. The vessel is rocked in a thermostatically controlled water bath by a mechanical shaker. B. Mass spectrometer leak assembly. Front and side views of the all-metal assembly are depicted. Copper capillary tubing is pinched at L by bar clamped against backing plate P. Gas from circulating system (fig. 3) flows through tubing soldered into block B_1. The open end of the capillary leak tubing is inserted into this gas stream. Diffusion through the leak transfers the gas for analysis into the cavity of block B_2 through needle valve V_2 to the connection T in the spectrometer gas inlet tube. Valve V_1 connected to a mechanical pump permits evacuation of the region between the leak and valve V_2. Entire assembly mounted on one quarter inch brass plate bolted to mass spectrometer manifold supporting frame.

cates via a T-connection with the gas inlet tube of the mass spectrometer. This eliminates the need for the leak normally connecting the instrument manifold with the spectrometer tube. Therefore the high pressure side of the manifold leak is kept evacuated when the instrument is employed as described in this paper so that the mass spectrometer measures only the gas diffusing in through the new leak from the gas stream being circulated through the experimental vessel.

Experimental procedure

When carrying out an experiment with this apparatus the biological material is placed in whatever type of vessel is to be employed. The vessel and the accessory gas circulating system is flushed either with a prepared gas mixture or with a background gas and, after flushing ceases, small amounts of other gases are added as previously indicated. The gas mixture thus is prepared in the apparatus and can be analyzed *in situ* so that, by trial and error, the desired gas mixture may be approximated. In most cases this requires about 10 to 15 minutes. Equilibration is rapid. Metabolic data usually can be considered meaningful about 15 to 30 minutes after the experimental vessel is placed on the apparatus.

Interpretation of data

After the experiment the strip chart must be analyzed to convert a recurring peak pattern, such as figure 2 B, into a more conventional type of graph. Data are taken off the chart by measuring the various peak heights (correcting for base lines) and making the appropriate corrections for amplifier sensitivities. The resulting data are relative and can best be converted to absolute values by direct calibration with known gas mixtures. It may also be noted that a given partial pressure of one gas will not necessarily produce the same mass spectrometer voltage output as the same partial pressure of a different gas. This is because the ionization efficiencies of different gases may differ significantly, hence the need for individual calibrations when absolute values are wanted. Another point to be remembered is that it is infeasible to employ the usual corrections for spectrometer residual peaks (the peak heights obtained when the mass spectrometer inlet valve is closed so that no gas can enter the tube). Apparently it is the presence of water vapor in the metabolic gases which invalidates the usual type of blank correction. Since there is no provision for drying the gas being analyzed, the apparatus is far from clean in the sense this term is used in mass spectrometry. Water vapor has not constituted a source of difficulty in our experiments. If it should prove objectionable in future applications, a drying agent or cold trap may be introduced in the gas circulating system in front of the T-connection to the mass spectrometer leak.

Time resolution

Figure 5 A shows a continuous record of the mass 32 peak when the apparatus was filled initially with helium. Time runs from right to left on the chart. As described previously, a section of the gas circulating system isolated between two stopcocks was filled with 100% oxygen. The stopcocks were turned so as to allow gas circulation, the pump was started, and the slug of oxygen was swept around the system again and again until complete mixing had been accomplished. The instrument detected this oxygen each time it passed the connection to the spectrometer leak tubing giving rise to the oscillating record shown in figure 5 A. The time of one cycle on

the chart corresponds to the circulation time of the gas in the apparatus and is 24 seconds under the particular conditions of this measurement. The time for complete mixing is about two minutes but this obviously depends on the size of the experimental vessel. With a larger vessel the mixing time would be less. Figure 5 A gives some indication of the time resolution achieved by the method.

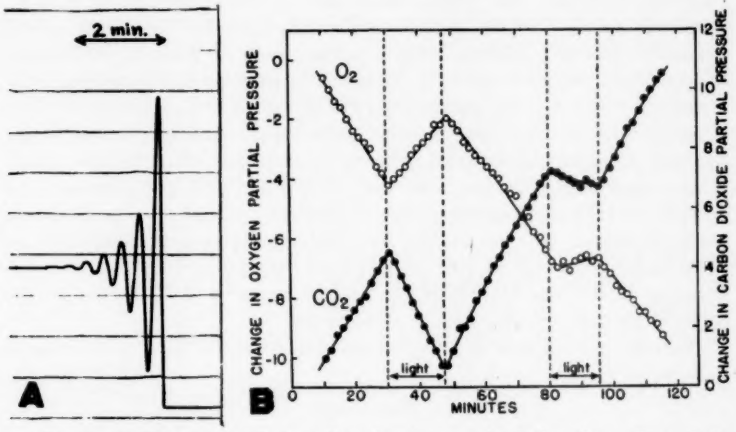

FIG. 5. A. Photograph of chart data showing gas circulating time. Part of the system (fig. 3) was filled with oxygen and the mass spectrometer was tuned to mass 32. The gas circulating pump was started and the gas was completely mixed after six circulations past the spectrometer leak. From this record, circulation time was 24 seconds. The measured system volume was 51 ml. The calculated pumping rate was 128 ml. per min. B. Sample data illustrating mass spectrometric measurement of respiratory and photosynthetic gas exchange of *Chlamydomonas Moewsii*. One hundred microliters of algae were suspended in 2 ml. phosphate buffer, pH 5.4. The gas phase was 3.5% oxygen in helium. Three dark and two light periods are represented. First light period gave photosynthesis 2 × compensation; second light period was near compensation. Points on each curve represent intervals of 108 seconds. The units on the ordinate correspond approximately to 10 microliters of either gas.

Data

After appropriate analysis of the data from the strip chart one may draw a graph of the gas exchange of the cells or tissue observed in the usual manner. Figure 5 B is an example which is chosen merely as an illustration. The biological material was 2.0 ml. of a 5% algal suspension in a rectangular vessel of 10 ml. volume (similar to figure 4 A) shaken in a water bath. The gas phase consisted initially of helium containing about 3% oxygen. The data show two intervals of photosynthetic activity at different light intensities between dark intervals during which only respiratory gas exchange is recorded. The ordinate values have no absolute significance; they have the dimensions of voltage output. By calibration with gas mix-

tures they can be converted to partial pressure increments of the respective gases. Such values can in turn be converted to absolute quantities of gases produced or consumed only if the total gas volume is known. In the experiment illustrated each unit on the ordinate corresponds roughly to 10 microliters of either gas.

Extreme fluctuations in the record typically are about 1% or less. Obviously the precision with which increments in partial pressure of a given metabolic gas can be measured thus depends upon the prevailing partial pressure. This is in contrast with a method such as Warburg manometry for which the precision neither is improved by working at low concentration of the gas which is being metabolized nor is there any sacrifice in precision if one works at high concentration. Practical considerations including quantity of biological material studied, its metabolic rate, the gas volume of the experimental system, and the size of the mass spectrometer leak actually determine the effective upper limit to the partial pressure of the gas being measured. We have taken data on rates of oxygen metabolism by plant tissues in as high as 10% oxygen with a statistical error in rate determination of less than 5% (standard error of regression coefficient of partial pressure on time for data such as those of fig. 5 B).

Various applications of this method will be dealt with in subsequent papers.

Summary

A method is described for measuring gaseous metabolism of cells, tissues, or whole organisms. In principle it consists of an experimental chamber of suitable size either with or without provision for gas circulation according to the requirements of the experiment. The gas phase is continuously sampled by a recording mass spectrometer. This instrument is provided with automatic tuning in such a manner that it records in sequence the partial pressures of several gases in the chamber at frequent intervals. The device accomplishes practically continuous monitoring of a predetermined number of gases. The analyses are of course based on the molecular weights of the respective gases and therefore the method has excellent analytical specificity except for a few ambiguities (like nitrogen and carbon monoxide). The method has good time resolution. Sample data are presented illustrating application of the method in a biological experiment.

Many times during the development of this method a number of individuals have contributed useful advice, helpful suggestions, or manual assistance in ways too numerous to mention in detail. Among those especially to be thanked are Charles Bollenbacher, Jaan Jurrisson, and J. M. Daly. We are particularly grateful to Dr. R. E. Halsted who was responsible for the basic electrical circuit design, essentially similar to that shown in figure 1, and who has advised us on many technical aspects of mass spectrometry. Kent Chapman and Wilma Monserud prepared most of the figures.

This work has been generously supported by grants from the Graduate School and from the Rockefeller Fund. Part of the development was carried out under contract with the Office of Naval Research (Contract no. N8onr-66208). Enrichment of the oxygen used in the experiments from which sample data were taken was made possible by a grant from the American Cancer Society through the Committee on Growth of the National Research Council. One of us (R.W.V.) wishes to acknowledge support in the form of an Atomic Energy Commission Predoctoral Fellowship in the Biological Sciences. During part of the time this method was being developed, the senior author held a Graduate School Summer Research Appointment. This encouragement is gratefully acknowledged.

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NITROGEN AND PHOSPHORUS FRACTIONS, RESPIRATION, AND STRUCTURE OF NORMAL AND CROWN GALL TISSUES OF TOMATO

RICHARD M. KLEIN

(WITH ELEVEN FIGURES)

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Introduction

The development of masses of tumorous tissue following implantation of *Agrobacterium tumefaciens* into susceptible plants has been of considerable interest to biologists for half a century. Among the many studies on these crown galls, few, however, have attempted to compare and correlate the structure and vital activities of normal and tumorous tissues from the time of inoculation through the various phases of development of the normal control plant and of the tumorous plant. For the tomato plant, RIKER (**24, 25**) has made sequential histological and cytological examinations of these tissues; RIKER *et al.* (**26**) and LINK and EGGERS (**15**) have studied the auxin levels; and LINK and GODDARD (**16**) have made a preliminary investigation of the respiration of the control and tumorous hypocotyl tissues throughout the period of tumor development.

The work reported in this paper was undertaken to compare some of the biochemical properties of normal and tumorous tissue and correlate these findings with the modifications in structure that occur during development. Nitrogen and phosphorus fractions and respiratory intensities were selected for this study because of their well-known relationships to energy and growth metabolism.

Materials and methods

Approximately 6,000 tomato seeds (*Lycopersicon esculentum* Mill. var. Bonny Best) were sown into 10 flats of soil on June 6, 1950. When the seedlings were 22 to 24 days old they were selected for uniformity and transplanted into flats (100 plants/flat) or into five-inch pots (one plant/pot). The 4000 plants so selected were divided into two lots, each occupying half of an east-west facing greenhouse room. On July 5, 1950, 24 days after germination, the plants of one lot were inoculated in the middle of the aboveground portion of the hypocotyl with a 24 hour old nutrient-dextrose broth culture of *Agrobacterium tumefaciens* (Univ. of Chicago strain S 5–6). The composition of the medium and the method of inoculation was that of BRAUN (**2**) and all of the inoculated plants developed tumors. The plants of the control lot were similarly wounded in the middle of the hypocotyl with a sterile needle. The hypocotyl was selected for this study because it

is the first axis segment to complete elongation and because of its relatively uniform diameter and stage of development under uniform cultural conditions (**15**).

Two days before inoculation and at time intervals of 2 to 44 days after inoculation, samples were collected for analysis. After the 44th day the plants began to flower and, as previously noted by STAPP and BORTELS (**31**), the tumors no longer grew after the plants were in bloom. All collections were made at 9:00 A.M. on the designated days. The plants in flats were used only for the first two collections when very large numbers of plants were required; thereafter the potted plants were used exclusively. For the first two collections after inoculations only the water-soaked tissues surrounding the punctures were used since this zone apparently delimits the prospective tumorous area (**23**). When the tumors were macroscopically evident only the enlarged portion of the hypocotyledonary cylinder was examined. Control segments routinely included the middle third of the hypocotyl. The selected hypocotyl segments were removed with a razor blade and rinsed with running tap water. During the earlier collection periods tissues from at least 150 control or tumorous plants were used and a minimum of 75 plants were sampled in all later collections. Tissue from the same plant was used for all of the studies reported here.

NITROGEN ANALYSES

Four to six gram samples of fresh tumor or control hypocotyl tissues were placed in weighing bottles, and, after determining their initial weight, were heated at 110° C for two hours. The tissues were then dried to constant weight in a vacuum oven at 60° C (**17**), and the percentage dry weight was calculated from these data. The dry samples were ground in a Wiley mill to pass a 60 mesh screen and stored in the dry state until analyzed. Total nitrogen was determined by the semi-micro Kjeldahl method using selenium as the digestion catalyst. Soluble nitrogen was extracted from the dry tissue for 18 hours at room temperature by 55% ethyl alcohol containing 5% trichloroacetic acid and four subsequent extractions were made at 80° C for 20 minutes each. Aliquot portions of the pooled soluble fraction were analyzed as for total nitrogen. The insoluble residue contained no alcohol-soluble nitrogen after the final extraction. Protein nitrogen was determined by difference and all data were referred to both wet and dry weight bases. The percentage standard deviations averaged 3%.

PHOSPHORUS ANALYSES

Several modifications were introduced into the phosphorus fractionation scheme develop by JUNI *et al.* (**10**) and the revised procedure is given here in detail. This method has the advantage of requiring a short time for a complete analysis and may prove useful in general surveys of the phosphorus metabolism of higher plants. Total phosphorus was measured after digestion of the plant tissue at 140° C for 18 hours in open tubes with 10 *N*

H_2SO_4 and concentrated HNO_3 and aliquot portions of the wet ash were analyzed by a modification of the FISKE and SUBBAROW method (**9**). The reduced phosphomolybdate color was fully developed within five minutes by including ethanol to a final concentration of 10% by volume (**28**) and was read in a Klett photoelectric colorimeter with the red filter.

Weighed samples of fresh tissue (1 to 1.5 grams) were placed together with fine sand in small glass mortars containing a frozen paste of 10% trichloroacetic acid and thoroughly ground. The mortars were kept at 4° C for two hours with occasional regrinding following which the samples were quantitatively transferred to centrifuge tubes using small volumes of cold 5% trichloroacetic acid as rinse fluid. After cold centrifugation the residues were re-extracted four times with cold 5% trichloroacetic acid, the pooled extracts made alkaline (pH 8.2) with NH_4OH, $MgCl_2$ added to a final concentration of 4% and the extracts stored overnight at 4° C. No additional acid-soluble phosphorus was extractable upon further treatment with cold trichloroacetic acid. The inorganic phosphorus in the extracts had precipitated by the next morning and was separated from the acid-soluble organic compounds by centrifugation. Preliminary trials showed that no phosphorus-containing esters were precipitated by this treatment. The precipitates were washed four times with small portions of dilute (1:10) NH_4OH, and the washings were added to the acid-soluble organic fraction. Aliquot portions of the pooled acid-soluble organic fraction were wet ashed for total phosphorus. This fraction was not further characterized. The inorganic phosphorus was analyzed by the methods outlined by JUNI *et al.* (**10**), metaphosphate being precipitated at pH 4.5 to ensure the maximum yield.

The residue remaining after the extraction of the acid-soluble fractions was dried at 65° C, transferred to soxhlet extraction thimbles, and extracted for two hours with ethyl ether (25 to 30 complete extraction cycles). The ether extract was analyzed for lipid phosphorus. The fat-free residues were then extracted four times with 5% trichloroacetic acid for 10 minutes at 90° C and the extracts pooled to form the nucleic acid fraction. This procedure (**29**) apparently removed only nucleic acids since no other phosphorus-containing compound was detected. Desoxypentose was determined using diphenylamine reagent (**8**) with the resulting color checked for absorbtion maximum, and the desoxyribonucleic acid (DNA) was calculated as a tetranucleotide from standard curves prepared by the method of SCHNEIDER (**29**). Since, as reported by OGUR and ROSEN (**21**), hot trichloroacetic acid treatments will hydrolyze pentosans, the standard pentose tests are unreliable as measures of ribonucleic acid in plant tissues. For this reason ribonucleic acid phosphorus and ribonucleic acid tetranucleotide (RNA) were calculated as the difference between the total nucleic acid phosphorus and the DNA phosphorus. Inasmuch as any determinate errors in the diphenylamine test would affect the observed concentrations of both nucleic acids, this method may not be completely accurate. However, this modification of the Schneider procedure was subsequently compared with the OGUR and ROSEN

(**21**) method with excellent agreement between the two techniques with regard to separation and analysis of the nucleic acids of tomato stem tissues.

The fat-free and nucleic acid-free residues were wet-ashed and analyzed for phosphorus. This fraction contained only protein-bound phosphorus.

In preliminary trials during the development of this phosphorus fractionation scheme, the average percentage standard deviation was 2.5%. Recoveries of added phosphorus compounds to plant tissues was 96% to 103% with an average recovery of $\pm$ 3% from theory. In the studies reported at this time it was impossible to run a sufficiently large number of replicate analyses at any collection period to calculate standard errors. In an effort to ensure reasonable dependability of the results, tissue fragments from at least 75 plants were pooled at each collection and all fractionations were run in duplicate. In no case did the duplicates vary more than 5% from their mean. The sum of the phosphorus in each fraction was usually 3% to 5% below the total phosphorus values and in view of the number of transfers and dilutions, this does not seem to be excessive.

Manometric methods

Tumorous and control tissues were cut free-hand into thin (0.5 to 0.8 mm.) slices with a razor blade and transferred to water at room temperature. After all the tissue had been sliced the water was filtered off through a porcelain filter. This rinsing of the tissue removed most of the bacteria (**20, 27**) and also served to randomize the slices from a number of plants. The buffer consisted of half strength Kreb's Ringer solution (to supply ionic cofactors) + 0.01 M potassium phosphates adjusted to pH 5.3 for respiratory study and pH 6.0 for the study of fermentation. Hydrogen or nitrogen gas, previously passed over heated copper filings to remove oxygen, was bubbled through the fermentation buffer for 10 minutes before it was used. Approximately 600 mg. of fresh tissue and 3.0 ml. of the appropriate buffer solution were placed in the main compartments of 25 ml. Warburg vessels. Respiratory gas exchange was measured by a one-vessel modification of the Warburg direct method (**13**) with air as the gas phase. Fermentation (anaerobic respiration) was measured in oxygen-free nitrogen or hydrogen and any bound CO_2 was released by acid tipped in at the end of the run. All runs were made in duplicate at 28° C. Respiration and fermentation were followed for 120 and 90 minutes respectively. All tissues were dried at 110° C to constant weight. The duplicate vessels agreed to within 10% on a dry weight basis.

Results

Histological findings

Free-hand sections of normal and tumorous hypocotyl tissues made at each collection showed the same sequence of histological changes noted by Riker (**24**). Only rare cell divisions were noted in control or prospective

tumorous tissues during the first four to five days after puncture or inoculation. At the end of this time cells about the point of the wound were beginning to divide. This period is designated as the induction phase (IP) and is so indicated on all figures. The control plant sections showed that the number of new cells formed in the puncture zone subsequent to the induction phase was small and they were restricted to the periphery of the wound. Cell division stopped about eight days after wounding. These new cells simulated a wound phellem in that the cells were tabularly arranged but they did not show extensive cell wall suberization. In the inoculated plants, however, cell division continued and did not slow down until about 20 days after inoculation. This period of rapid tumorous cell proliferation is designated as the growth phase (GP). These tumorous cells do not constitute a typical callus because the cell divisions, which take place in all planes, were not preceded by extensive cell enlargement. During the growth phase the only new cells formed in the control hypocotyl tissues were those cut off by normal cambial activity, and the maturation of the control tissues proceeded in normal fashion.

Fourteen to 20 days after inoculation the tumor consisted almost entirely of small meristem-like cells, and it appeared that by this time the maximum number of tumorous cells per unit weight or volume of tissue had been formed. The third phase of tumor development was initiated at this time and is designated as the maturation phase (MP). During this period tumorous cell division decreased rather sharply and had almost ceased by the end of the study. Large numbers of tumorous cells, instead of dividing, were enlarging and maturing. The walls were undergoing secondary thickenings, dead elements (sclerids, wound tracheids, etc.) were seen, and necrobiotic changes were evident in the protoplasts. Nevertheless, the proportion of young cells was still considerably higher in the tumorous tissue than in the control tissue. In the control tissue, the number of new cambial derivatives decreased and maturation of the several tissues continued up to the end of the study.

Weight changes

Nagy *et al.* (**19**) and Link and Eggers (**15**) did not find any significant differences in the percentage dry weight between tumor and control at the time of their collections of material. In the experiments reported at this time, there was a distinct difference between these tissues after the fourteenth day of tumor development (fig. 1). Although the percentage dry weight of the tumorous tissues remained constant after this time, that of the control increased steadily until the close of the study. These differences in dry weight have been reported for old tumors and their controls by Klein and Keyssner (**11**) and would be expected if, as noted above, the tumor consisted of young cells while the control tissues showed considerable secondary thickening. It should be remembered, however, that these differences might not be observed if the control tissues remained quite succulent

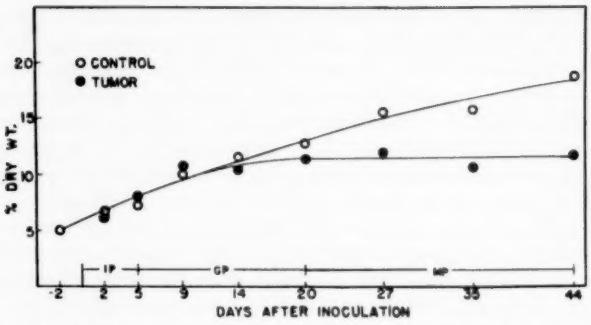

FIG. 1. Changes in the percentage dry weight of control and tumorous tomato hypocotyl tissues during tumor ontogeny.

throughout the period of study. The relation of the dry weight to the calculations of gas exchange rates and to the levels of tissue constituents will be discussed later in this paper.

NITROGEN CHANGES

The relation of soluble and protein nitrogen to the time after wounding or inoculation is illustrated in figure 2. If there were any nitrogen changes during the induction phase in response to the wounding of the control plants, the changes were either too small to be detected by the analytical methods used, or the samples were too large to uncover purely local changes. The shapes of the curves for the protein and soluble nitrogen of the control tissues are typical of the changes in concentration to be found in normal tissues with increasing age when they are calculated to a dry weight base. When plotted on a fresh weight base, the curves showed a slowly rising level of nitrogen (or other) fraction with time.

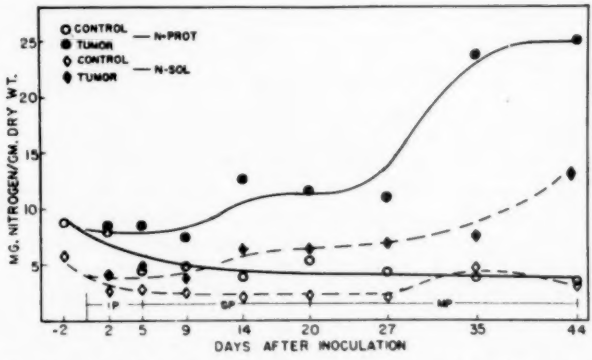

FIG. 2. Concentrations of protein and soluble nitrogen in control and tumorous tomato hypocotyl tissues during tumor ontogeny.

The major increase in protein nitrogen of the tumorous tissue occurred relatively late in development and appeared to be initiated at the time when the tumor changes from primarily numerical cell increase to cell volume increase. The soluble nitrogen showed the same pattern but the rate of accumulation was smaller. Prior to the present study, only the 35 to 44 day stages have been used for the reported differences in the nitrogen content of normal and tumorous tomato tissues (**11, 19**).

The relative proportion of protein to soluble nitrogen constituents during development is of considerable interest. KLEIN and KEYSSNER (**11**) reported a predominance of protein nitrogen in tomato tumors five to six weeks after inoculation while the opposite was true for control tissues. This appears to be true only during the period of tumor maturation since protein was not being preferentially synthesized during the main period of tumor growth. Since soluble nitrogen also increased in the tumor during the maturation phase, albeit at a slower rate than protein nitrogen, the synthesis of protein did not occur entirely at the expense of the available amino acids and amides. The stunted and nitrogen-deficient appearance of the tumorous plants would lead one to postulate that the nitrogen of the whole plant was being mobilized into the tumor.

NUCLEIC ACID CHANGES

The variations in the level of DNA in tumorous tissue during the period of study were quite striking (fig. 3). Two days after inoculation there was a peak in the level of DNA with no evidence of cell division about the point of inoculation. The recent work of SWIFT (**32**) suggested the possibility that there may be a duplication of chromatin in these pre-tumorous cells since in normal plant tissues the DNA per nucleus doubles preceding cell division. As no corresponding change was noted in the wounded control tissues, this increase in DNA may be the first indication of the subsequent burst of cell division noted during the early growth phase. However, the subsequent drop in the concentration of DNA is not consistent with this duplication hypothesis since, unless DNA was being destroyed, the curve should either form a plateau at this level or increase until the second peak was reached. It is possible that this initial, high DNA level represents some yet unknown activity of the pre-tumorous cells which may be directly related to the processes of tumor induction. The second peak in DNA level of the tumorous tissue is almost certainly due to the large numbers of cells per unit weight of tissue. Measurements of the relative amounts of DNA per normal and tumorous cell nucleus by the methods of SWIFT (**32**) at the time of the second peak (14 days after inoculation) did not show any significantly higher concentration of DNA in the tumorous cell nucleus as compared to the control (H. H. Swift, unpublished). The histologically observed enlargement and maturation of the tumorous cells subsequent to the growth phase would explain the drop in the DNA level since the number of cells per unit weight of tissue appeared to have decreased after the 14 to 20 day peak.

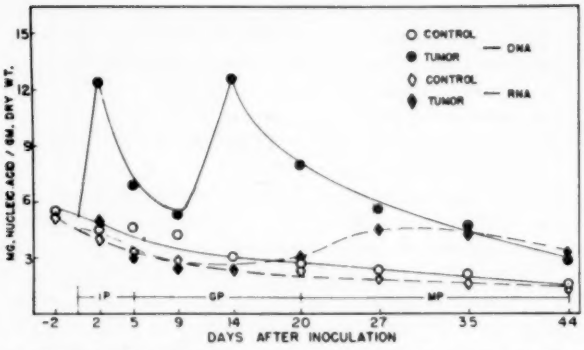

FIG. 3. Concentrations of desoxy- and ribonucleic acids in control and tumorous tomato hypocotyl tissues during tumor ontogeny.

Since the concentration of RNA in the tumorous tissues followed that of the control tissues during the induction and growth phases in spite of the obvious increase in cell numbers, it would appear that the cellular concentration of this nucleic acid does not remain constant during the period of tumorous cell multiplication (fig. 3). The rise in the RNA concentration in the tumorous tissue 27 to 35 days after inoculation occurs at the time protein accumulation is greatest and cell division had largely been supplanted by enlargement and maturation. This association of changes in the concentration of RNA and protein has been postulated as a causal relation by CASPERSSON (**6**) but has not been reported in higher plants. This problem will be discussed in another section of this paper.

The ratio DNA/RNA of the control tissue was approximately 1.0 to 1.2 throughout the mature life of the plant (fig. 4). Another indication of the constancy of a ratio of this type can be noted in the nucleo-cytoplasmic ratio of normal tissue as reported by RIKER (**26**) although the specificity of DNA and RNA in plant nuclei and cytoplasm respectively has not been established. In the tumor the ratio varied, reaching a peak of 4.8 at the end of the growth phase. The fall in the nucleic acid ratio during the mat-

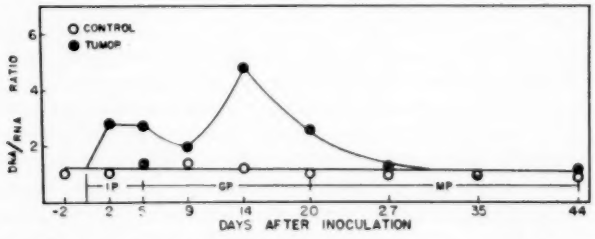

FIG. 4. Changes in the ratio: DNA/RNA of control and tumorous tomato hypocotyl tissues during tumor ontogeny.

uration phase to that found in the control is, to some extent, a biochemical confirmation of the histologically observed maturation of the tissues. It is doubtful, however, whether this normal ratio indicates that the tumor can now be compared to controls of the same chronological age.

Phosphorus changes

The peak in the accumulation of total phosphorus in the tumorous tissue was observed during the late growth phase (fig. 5). This piling up of total phosphorus in crown gall tissues has been studied recently by Tsao and Whaley **(33)** using phosphorus 32 accumulation in Bryophyllum tumors. These authors also reported higher concentrations of total phosphorus in

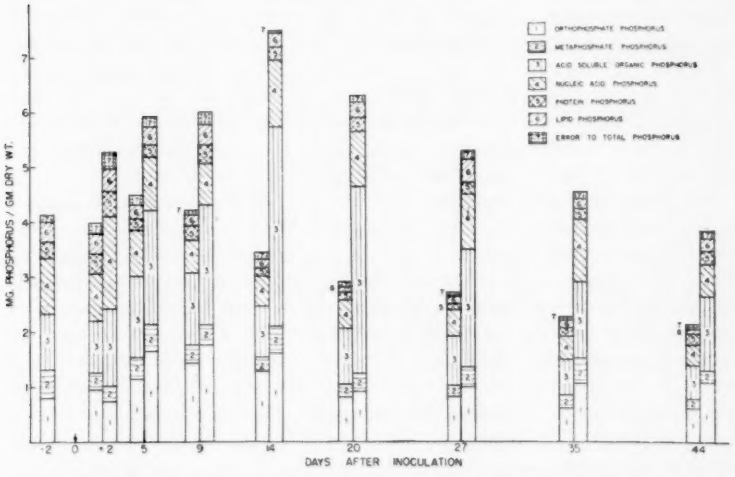

Fig. 5. Concentrations of various phosphorus-containing tissue fractions of control and tumorous tomato hypocotyl tissues during tumor ontogeny. Single bar at 2 days before inoculation (−2 days) represents normal hypocotyl tissues only. Left bar at all collection times represents fractions in control tissues; right bar represents tumorous tissues.

tomato stem tumor tissues as compared to normal tissues, their values closely approximating those reported here. When the total phosphorus values reported in the present study were calculated on a DNA base rather than per unit tissue weight in an effort to determine the relative concentrations of phosphorus per cell, the differences in the levels of phosphorus in control and tumorous tissue disappeared and the time course curves were practically superimposable.

The orthophosphate phosphorus content of the control tissue comprised about 25% of the total tissue phosphorus throughout the period of study. In the tumor, however, during the late induction and early growth phases,

there was some indication that the orthophosphate phosphorus became a somewhat greater fraction of total phosphorus than was true of the corresponding control. The orthophosphate contribution to the total phosphorus of the tumor then decreased to about 20% during the growth phase, and the proportion again rose to the control value towards the end of the maturation phase.

The presence of metaphosphate has been reported in fungi (*Aspergillus, Neurospora,* and yeasts) but has not previously been noted in the tissues of higher plants. The compound (or polymer) has not yet been assigned any specific role in metabolic processes. In the hypocotyl tissues of the controls, metaphosphate phosphorus remained a fairly constant fraction of the total phosphorus throughout the period of study. In the tumorous tissues, its relative importance decreased markedly as the total phosphorus content of the tissues rose. Whether this indicates that this little-studied fraction is of slight importance in the phosphorus metabolism of proliferating plant tissues or that it serves as a stable pool for other phosphorus-containing compounds must await further study. In view of the increase in cell numbers in the tumorous tissues, it is certain that metaphosphate did not remain constant in amount in tumor cells as compared to control levels.

The remarkable increase in the components of the acid-soluble, organic phosphorus fraction of the tumorous tissue is of considerable interest. During the late growth phase of tumor development this fraction, containing phosphorus esters, acid-soluble nucleotides, etc., comprised more than half of the total tissue phosphorus. When calculated to a DNA base instead of weight, there is good reason to infer that these components increase in amount per cell. It is doubtful whether this increase is due entirely to a block in the Meyerhof-Embden pathway since no decrease in the rates of tumorous tissue fermentation was observed. During the late maturation phase of tumor development the relative role of these compounds decreased until, at the end of the study, they formed approximately the same fraction of total phosphorus as was found for the controls. The detailed fractionation scheme developed for the analysis of these components by LEPAGE and UMBREIT (**14**) would be most useful in an analysis of this problem but could not be used in the present investigation.

Although the nucleic acid phosphorus and lipid phosphorus fractions remained reasonably constant as percentages of total phosphorus in both tumorous and control tissues during the period of study, there were small increases in the percentages of tumorous tissue protein phosphorus during the growth phase of development. Here the time dependent changes in the level of this component appeared to be more closely related to DNA (and hence cell numbers) than to the weight of the tissue. The rise in protein phosphorus of the tumor during the early induction phase, two days after inoculation, which is unrelated in increases in cell numbers, may be significant since BRAUN (**3**) has postulated that the tumor inducing principle may be protein in nature.

Gas exchange

The relative constancy of the respiratory quotient (R.Q.) of control hypocotyl tissues close to unity would indicate that throughout most of the mature life of this organ the primary respiratory substrate is carbohydrate (fig. 6). This corroborates the finding of Caldwell and Meiklejohn (**5**). In the present study the R.Q. was 0.92, 27 days after planting (22 days after seed germination) and then the R.Q. levels off at 0.97 to 0.99 when the plants were in the four to six leaf stage, 28 days after germination. The progressive modifications of the R.Q. of the slices of tumorous tissue suggests that the substrate for respiration was changing. The initial drop to 0.85 immediately after inoculation might indicate that protein was being oxidized but no data are available at this time to evaluate this finding. It is also possible that this R.Q. change is more closely related to an incomplete oxidation of carbohydrate with an accumulation of its end products (organic acids, etc.).

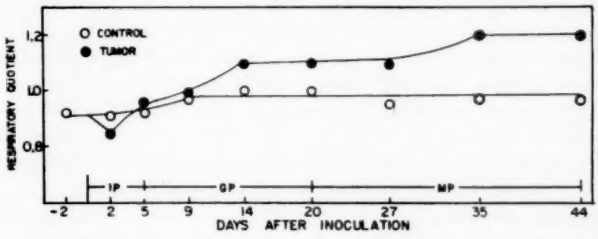

Fig. 6. Respiratory quotient of slices of control and tumorous tomato hypocotyl tissues during tumor ontogeny.

The subsequent rise in the R.Q. to 1.2 during the maturation phase would suggest the utilization of compounds in a higher state of oxidation than carbohydrate. Although the nature of this substrate is unknown, it is of interest that Klein and Keyssner (**12**) reported that five to six week old tomato tumors were more alkaline than control tissues and contained more titratable acidity. This suggests that there might be an accumulation of organic acids in the tumorous tissues which might be available for respiratory utilization. It should be remembered, however, that this high R.Q. may reflect a concomitant aerobic glycolysis of the tumorous tissues; but again, no convincing evidence is available.

The rates of gas exchange of control and tumorous tissue slices were plotted on weight, nitrogen, phosphorus, and nucleic acid bases in order to compare these standards as to significance and to gain an insight into the types of response of these tissues during their various phases of development. It must be emphasized that the gas exchange rates of isolated tissues cannot be assumed to represent the activities of these tissues in the environment of the intact plant.

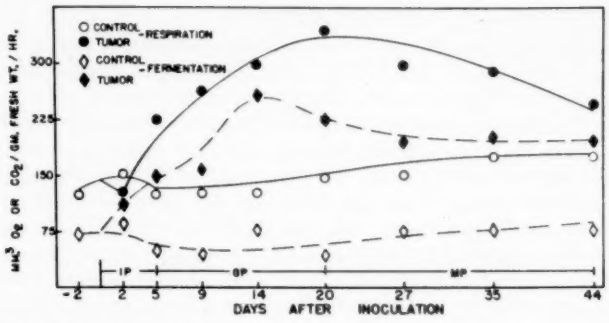

FIG. 7. Respiration and fermentation of slices of control and tumorous tomato hypocotyl tissues during tumor ontogeny when plotted on a fresh weight basis.

The curves for oxygen uptake (respiration) and anaerobic CO_2 production (fermentation) on fresh and dry weight bases (figs. 7 and 8) show that the gas exchange rates of tumorous tissues were considerably higher than those of the controls with the peak in respiration occurring at about the end of the growth phase. On both these bases there were small but significant decreases in tumor respiration immediately after inoculation and a slight increase in fermentation at the same time. The curves for oxygen uptake on a fresh weight base are quite similar to those reported by LINK and GODDARD (**16**). However, these authors did not find any decrease in oxygen uptake upon tumor initiation when they calculated rates on a fresh weight base. The steady decreases in gas exchange rates of control tissue slices calculated on dry weight standards is primarily a function of the increments of non-respiring elements (vessels, fibers, cell walls, etc.) which make up

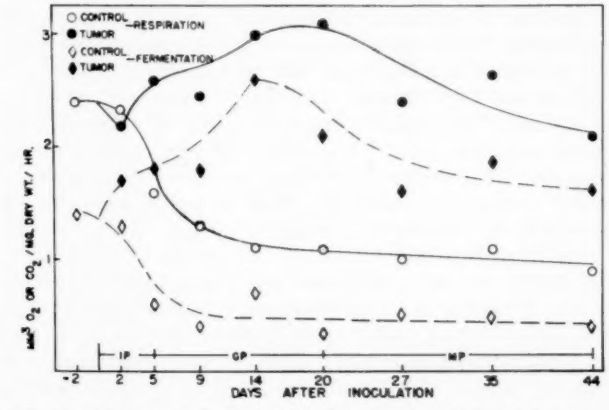

FIG. 8. Respiration and fermentation of slices of control and tumorous tomato hypocotyl tissues during tumor ontogeny when plotted on a dry weight basis.

increasingly greater fractions of the dry weight with increasing age of the tissues. When fresh weight was used as the reference standard, the ascending control curves during the period of study are related more to the changing number of cells per unit volume of tissue since the dead material, although increasing in amount, comprises a smaller fraction of the fresh weight than of the dry weight.

Nitrogen bases have been suggested and used for the calculation of the gas exchange rates of plant tissues because of the ease of determining this cell fraction and because of the assumption that nitrogen will represent living material. These views have been discussed by BURRIS and WILSON (**4**). The gas exchange rates of control and tumorous tissue slices have been plotted for total nitrogen (fig. 9), soluble nitrogen, and protein nitrogen. There was good agreement in the shapes of the curves when total and protein nitrogen were used as standards for calculation. The respiration of the

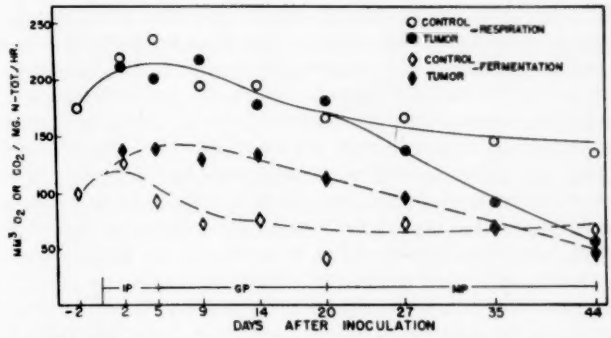

FIG. 9. Respiration and fermentation of slices of control and tumorous tomato hypocotyl tissues during tumor ontogeny when plotted on a total nitrogen basis.

tumorous tissue was identical to that of the control during the induction and growth phases after which the respiration of the tumorous tissue fell off sharply. It will be remembered that this was the time that the tumor was maturing and nitrogen, particularly protein nitrogen, had started to accumulate. On the other hand, the rates of fermentation remained higher in the tumor throughout most of the period of examination, descending to the control level only at the end of the study. If these were the only data considered, it would appear that the fermentation of tumorous tissue was accelerated while respiration was unaffected during the induction and growth phases, falling off during the maturation phase. When soluble nitrogen was the standard, the picture was more like that seen on weight bases.

Various phosphorus fractions were also used for determining gas exchange rates. When the rates of control and tumorous tissues were plotted to a total phosphorus base (fig. 10) it can be seen that both the respiratory and fermentative capacities of the tumorous tissue parallel those of the con-

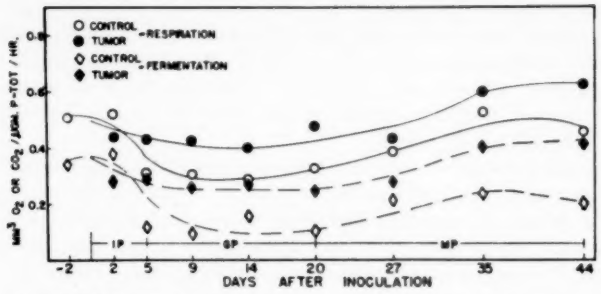

FIG. 10. Respiration and fermentation of slices of control and tumorous tomato hypocotyl tissues during tumor ontogeny when plotted on a total phosphorus basis.

trol tissues but are at somewhat higher levels. The striking similarity of the total phosphorus based curves to those employing a DNA standard (fig. 11) is particularly important. Since it was reported that DNA is reasonably constant per tomato cell nucleus, this nucleic acid should be an accurate measure of the number of living cells in any particular tissue sample. Therefore, these latter curves should compare the gas exchange rates of control and tumorous tissue on a more nearly cellular level than would be possible using any other biochemical characteristic of the tissue. Under the conditions of these studies (excluding the early induction phase values which are unrelated to cell numbers) the rates of gas exchange of tumorous tissue (tumorous cells) were parallel to but were greater than those of the control tissues (control cells) throughout most of the period of study. This elevated rate of gas exchange of the tumorous cell was initiated during the late induction phase, was maintained during the growth phase, and showed a further increase during the maturation phase. This secondary increase in gas exchange during the late maturation phase was also noted when total phosphorus was used as the reference standard. When protein phosphorus was used as the base, the gas exchange rates of the tumorous tissue were less

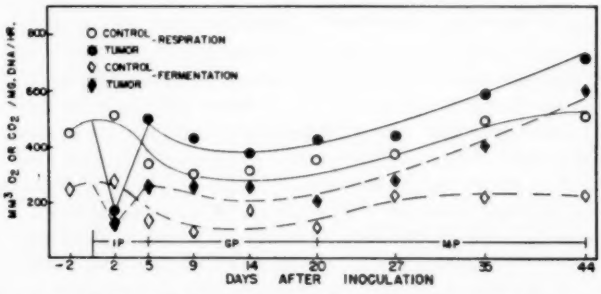

FIG. 11. Respiration and fermentation of slices of control and tumorous tomato hypocotyl tissues during tumor ontogeny when plotted on a desoxyribonucleic acid basis.

constant than those calculated to the other bases but the previously noted acceleration of the rates during the late maturation phase was evident.

Discussion

The patterns of the curves for gas exchange rates of the slices of control and tumorous hypocotyl tissues during the study varied considerably depending on the base used for the calculations. The problem remains as to the most adequate base for expressing gas exchange rates to reflect the metabolism of tissues which, in addition to being modified by normal aging processes, differ as a result of experimental procedure. If the concentration of DNA remains fairly constant per nucleus during most of the life of a cell and this nucleic acid is restricted to the nucleus, DNA would be the standard of choice. The use of nucleic acid standards for determining metabolic activities of small amounts of surviving tissue was suggested by BERENBLUM *et al.* (**1**) and used by DAVIDSON and LESLIE (**7**). The inadequacy of weight or nitrogen bases for this type of calculation is quite evident in a study of this kind in which there were definite weight differences during development of the control and tumorous tissues and nitrogen, particularly protein nitrogen, accumulated during the terminal stages of tumor development. Further, this accumulation of nitrogen, occurring at the time that DNA was decreasing in amount per unit weight of tissue, *i.e.*, the number of cells per unit weight were decreasing, indicates that the concentration of nitrogenous compounds per cell did not remain constant during tumor ontogeny.

Whether the striking correspondence in the sequential gas exchange rates of normal and tumorous tissues when based on total phosphorus and DNA constitutes evidence that total phosphorus was in the same concentration in control and tumorous cells must await further study. If this suggestion is borne out in future research, it may be possible to refer gas exchange (and possibly other metabolic activities) preferentially to a total phosphorus base, *i.e.*, $Q_{O_2}(P)$. This cell constituent is easily measured and determinations have the same accuracy as do those for nitrogen.

Gas exchange rates and nitrogen and phosphorus fractions, calculated to the various bases used in this study, were converted to percentages of the control as advocated by MITCHELL *et al* (**18**). Although the scatter of points was somewhat reduced, the results of these calculations are not reported at this time since the method of presentation does not permit rapid comparison with the results of other workers. Without exception the conclusions drawn from plots of these calculations were the same as those obtained by the more usual methods.

It is important to determine whether there were any specific and characteristic changes in gas exchange upon tumor initiation. The sharp rise in the DNA level at this time, which is unrelated to cell numbers, prevented comparisons on this basis. When weight, nitrogen, and total phosphorus data were considered, the respiration of the incipient tumorous tissue was somewhat depressed and fermentation was increased. Since it will be re-

membered that in this study the percentage dry weight and stages of development of control and incipient tumorous tissue were the same at this time, the conclusion that fermentation was increased and respiration decreased upon the initiation of the neoplastic state appears to be valid. As noted in the section on the R.Q., there was an even more pronounced depression in the rate of aerobic CO_2 production.

The biochemical behavior of the tumor during the induction phase is of particular importance since, as demonstrated by BRAUN (**2**), normal host cells have received maximum tumor-inducing stimulus during this period, some stimulation occurring even in 36 to 48 hours. Both protein and soluble nitrogen levels were elevated in this time and total phosphorus had also increased above the control level. Especially noteworthy were the increases in DNA and protein phosphorus of the incipient tumor. All of these modifications in the composition of the stimulated tissues occurring before cell divisions were evident. The relation of these changes and the modifications of gas exchange rates to the processes leading to the establishment of the tumorous state are, at present, unknown. In view of the high energy of inactivation of BRAUN'S tumor inducing principle (**3**), the increases in protein phosphorus, protein nitrogen, and DNA are suggestive.

The characterization of the tumor during the growth phase is most difficult. The respiration of the tumorous tissue (tumor cells) during this period is at a higher level than that of comparable control tissues (control cells) and the modifications in the R.Q. suggests that the primary respiratory substrate has become different than that of the control. The rates of tumor fermentation also were above control levels. The proportion of protein nitrogen to soluble nitrogen suggests that the dividing tumorous cell did not accumulate protein. No special changes were noted in the relative concentration of most of the phosphorus fractions although the production and/or accumulation of esters and acid soluble nucleotides was greatly accelerated. In attempting to evaluate the relations among protein nitrogen, RNA, and cell development, one is confronted by the lack of any direct experimental evidence for any more than a coincidental association of these factors in the tissues of higher plants. In a critical discussion of these phenomena, SPIEGELMAN and KAMEN (**30**) state: "While the experiments . . . show that protein synthesis or modification is associated with marked changes in nucleotide synthesis, none can be interpreted to exhibit a rigorous causal relation between the two processes." Thus it can only be stated that the association of these cellular components during the maturation phase of crown gall development parallels that found in other organisms. PETRIE (**22**) has reviewed the attempts to correlate protein synthesis in plants with changes in respiratory activity and concluded that, to date, these studies have not been marked with any great degree of success. Examinations of the curves relating to the changes in protein nitrogen of the tumorous tissue (fig. 2) and those concerned with the time-course of gas exchange calculated to total phosphorus (fig. 10) or DNA (fig. 11) standards show that at the time of

maturation of the tumor there were almost simultaneous increases in respiration and protein accumulation. This association of processes, too, cannot be definitely stated to be directed but is suggestive of more than a casual coincidence of a number of vital activities.

The significance of the sequential changes noted among the various factors and processes studied during the development of the control and tumorous tissues with regard to cause and effect are obscured by the lack of adequate information on their exact roles in the economy of the constituent tissues. Furthermore, the characterization of any of these developmental stages must be based on numerous structural, biochemical, and biophysical diagnostic aids since no one feature is capable of delimiting the facets of a growing, changing system or cell. Differences in structure, biochemical activity, and/or cell constituents are valid insofar as they are specifically related to a particular phase in the development of normal or pathological tissues. Therefore, the level or activity of any cellular character will be a function of the chonological and physiological age of the tissue and its stage of development in addition to the conditions imposed by the experiment.

Summary

1. In an effort to compare some of the biochemical properties of normal and tumorous tissues of the tomato hypocotyl and to correlate these findings with the modifications in structure that occur during the development of the tissues, a study has been made of the nitrogen and phosphorus compounds, the structure, and the respiratory intensities of these tissues from the time of the induction of the crown gall until the flowering of the plants when the tumors stopped growing.

2. During the first five days after inoculation or wounding of the tissues, *i.e.*, the induction phase of tumor development, only rare cell divisions were noted in either control or incipient tumorous tissues. No differences in the percentage dry weight were observed. By the end of the induction period, there were significant increases in both protein and soluble nitrogen fractions of the tumorous tissue but no modification of the ratio between these constituents. Immediately after inoculation, there were increases in the concentrations of desoxyribonucleic acid, and protein phosphorus in the inoculated tissues, and less marked increases in the concentration of the acid-soluble organic phosphorus compounds. In the wounded control plants, all nitrogen and phosphorus fractions examined showed the expected decrease in concentration when calculated on dry weight bases. Immediately after inoculation there was a slight depression in the rate of oxygen uptake of the pre-tumorous tissues as compared to the controls and a drop in the respiratory quotient from 0.92 to 0.85. There was, however, a pronounced increase in the rate of fermentation of the incipient tumorous tissues.

3. The period from the end of the induction phase until approximately 20 days after inoculation was marked structurally by rapid cell divisions in the tumorous tissue. By the end of this growth phase of tumor develop-

ment, there appeared to be the maximum number of tumorous cells per unit weight or volume of tissue. Cell divisions about the point of wounding of the controls had ceased a week after inoculation. The percentage dry weight of the control tissues continued to increase during this and the subsequent period of development but that of the tumorous tissues became established at a constant value by the end of the growth phase. There was some evidence of the accumulation of nitrogen in the tumorous tissue, although the ratio between protein and soluble fractions maintained itself at close to the pre-induction value. A peak in the concentration of DNA per unit weight of tissue was observed by the end of the growth phase but histochemical measurements indicated that the amount of this nucleic acid per tumorous cell nucleus was the same as that of the control. The production and/or accumulation of phosphorus esters and acid-soluble nucleotides was accelerated in the tumor during this period. The respiration rate of the tumorous cells paralleled that of the control cells and was at a higher level. The respiratory quotient of the control tissues was approximately 1.0 but that of the tumor rose to 1.2. The rates of fermentation of the tumorous cells paralleled those of the control and were also elevated.

4. The maturation phase of tumor development was structurally marked by tumorous cell enlargement and the formation of mature and dead elements. The drop in the ratio between the nucleic acids during this period to that found in the control is, to some extent, a biochemical confirmation of the histologically observed maturation of the tumorous tissues. There was an association among protein synthesis, ribonucleic acid, gas exchange, and cell maturation in that the concentrations and/or rates of activity of all of them increased at the same time.

This report is a continuation and extension of the studies on the metabolism of neoplastic plant tissues begun by Professor G. K. K. Link and is the third in a series of papers.

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PRE- AND POST-HARVEST CHANGES IN CAROTENE, TOTAL CAROTENOIDS AND ASCORBIC ACID CONTENT OF SWEETPOTATOES

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(WITH NINE FIGURES)

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In a preceding paper (**4**) data were presented showing the effects of storage temperature and variety on the carotene, total carotenoids and ascorbic acid content of sweetpotatoes. In this paper data regarding changes in these constituents during the harvest season, and the effects of the time of harvest on their behavior in storage are given.

Sweetpotatoes may be harvested at any time after they reach marketable size. They do not mature in the same sense as do apples, wheat or white potatoes, which have a more or less definite period of growth and then cease growing regardless of weather and cultural conditions. Cessation of growth may be due to lack of moisture or nutrients, to low temperatures or to other unfavorable growing conditions, but in the tropics or in a greenhouse sweetpotatoes may grow without interruption for years. The roots at harvest may therefore be growing vigorously, may have ceased growing entirely because of unfavorable growing conditions such as frost, or may be at any stage between these two extremes.

The metabolic activity of roots harvested when the plant is actively assimilating nutrients and laying down new tissues and reserve materials is doubtless different from that of roots harvested when growth has ceased and the root is dormant. The metabolic rate of the roots might be much higher after eight or ten days in the curing room than at the time of harvest. There the high temperature and humidity are favorable not only to the production of wound periderm, but also to the resumption of vegetative activity. The metabolic rate might be high during curing both in roots that had not been checked in growth before harvest and in those that had been checked, but the effects on the respective lots might be different. In the first case metabolic activity continues high throughout the period while in the second case the dormant root is stimulated into renewed activity after growth has ceased and life processes have slowed down. Activity at this time would probably stimulate development of adventitious buds and new shoots. In some cases sprouting occurs before the roots are harvested, probably as a result of a return of favorable growing conditions after a period unfavorable for growth.

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Some work regarding the effects of the time of harvest on the carotene content of sweetpotatoes has been done. ANDERSON *et al.* (**1**) reported that Porto Rico sweetpotatoes produced by late planting contained less carotene than those produced by early and midseason planting. KIMBROUGH *et al.* (**7**) varied both the planting dates and the times of harvest of Porto Rico. They concluded that in southern Louisiana planting dates up to the middle of June are not likely to affect the carotene content of roots of marketable size and that roots dug early may be as high or higher in carotene than those from the same planting dug later. EDMOND *et al.* (**2**) evaluated root color in the Porto Rico variety and concluded that in general the color of the flesh increased as time of harvest was deferred. EZELL and WILCOX (**3**) reported that carotene increased more rapidly with increasing depth of color of the flesh than did the non-carotene pigments. Changes in the ascorbic acid content of sweetpotatoes during the harvest season and the effect of time of harvest on the post-harvest behavior of carotene and ascorbic acid have not been reported previously.

Material and methods

Four varieties of sweetpotatoes were grown in 1949 on a Chillum sandy loam at the Plant Industry Station, Beltsville, Maryland, as described earlier (**4**). The varieties ranged in flesh color from the creamy white or light yellow of Yellow Jersey through the deeper yellow of Nancy Hall and Porto Rico (Unit I strain) to the deep orange of Orange Little Stem. Nine harvests were made extending from mid-August, when only a few of the largest roots were of marketable size, through November, approximately one month after frost had terminated growth. Only one variety was harvested on each day. The roots were washed, held overnight at 60° F and analyzed the next morning.

To determine the effect of time of harvest on the behavior of the carotenoids and ascorbic acid in storage, sufficient roots were harvested, cured and stored at the third (early), fifth (midseason) and seventh (late) harvests to permit periodic analyses during storage. The third harvests were made between September 6 and 15, approximately one month before the usual commercial harvest of sweetpotatoes for storage in this area, but at a time when the roots might be harvested for early market or home use. The midseason harvests, made between October 3 and 12, represented good commercial harvests before frost in this area. The late harvests were made between November 1 and 7, five to 11 days after frost had killed the foliage. Whether these three harvests represent a decreasing order in rates of growth and of metabolic activity is not known. It seems safe, however, to assume that appreciable differences existed in roots harvested approximately four weeks apart and that roots from the late harvests were less active than those harvested earlier.

Sweetpotatoes require a warm temperature. HARTER and WHITNEY (**6**) reported that sweetpotatoes plants held at 50° F turned a yellow sickly

color in three or four days and gradually died. At 59° F no growth took place but the plants were able to hold their own except for occasional yellowing of some of the lower leaves. Harter and Whitney concluded that the minimum temperature for growth of the plants was between 59 and 68° F. Even storage roots are injured if held continuously at 50° F or below. At the midseason harvest for storage a few of the leaves were beginning to turn a little yellow, which indicated less favorable growing conditions. However, the average daily mean temperature during this period was 67° F compared with 66° F at the early harvests. The late harvests were made after the foliage was killed by frost.

The methods and general procedure used in this study were the same as previously described (**4**). All lots were cured at 85° F for eight days and then stored in the same room at a constant temperature of 60° F. The carotene, total carotenoid and ascorbic acid contents for each sample as reported are based on duplicate determinations on each of ten replicates of five or more roots each, a total of 50 roots or more. The carotenoid pigments were calculated back to the weight at harvest and are reported on this basis. Ascorbic acid is reported on the fresh weight basis at time of analysis. Values departing significantly at the 1% level from the curves in the figures are marked with a double asterisk.

Results and discussion

Changes during the harvest season

The carotene, total carotenoid pigments, and ascorbic acid contents during the harvest season are shown in figures 1 to 4. Varieties differ in their manner of growth. Some varieties such as Yellow Jersey and Orange Little Stem form many miniature sweetpotatoes early in the season and these enlarge as the season advances. In other varieties such as Nancy Hall and Porto Rico a general enlargement along a main root usually takes place, resulting in a longer and heavier sweetpotato by the time a characteristic spindle shape is developed. In the early stages of enlargement such roots are often referred to as strings and these were not included in the samples.

At the first harvest few roots per plant were of acceptable size. The average weights of those used were 26, 36, 83 and 86 gm. respectively for Yellow Jersey, Orange Little Stem, Nancy Hall and Porto Rico. While these roots were small, it was not believed that the size of the root was of major importance in determining the carotenoid content. This was indicated in Orange Little Stem; the average weight of the roots used at the first harvest was 36 gm. A second sample of smaller roots, taken at the same time (average weight of 11 gm.) contained 93% as much carotene and total carotenoids per unit weight as did the larger roots. This small difference in carotenoid content between the two sizes was statistically highly significant, but since the larger roots were over three times as heavy as the smaller roots, size was not considered as a major factor in determining the carotenoid content even this early in the season.

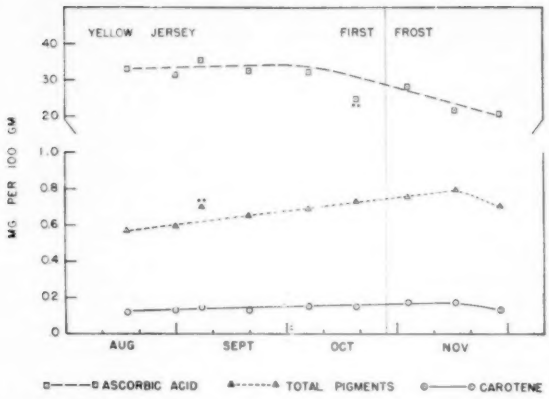

FIG. 1. Changes in the ascorbic acid, carotene and total carotenoid pigments of Yellow Jersey sweetpotatoes during the harvest period.

The harvest period covered in this study extended over a much longer period than is commercially feasible at this latitude. A grower digging even as early as our third harvest would be penalized heavily in yield, and few growers would dare leave roots in the ground long after frost had killed the foliage. The usual harvest period in this area is better represented by our fifth, sixth and seventh harvests, the period from the time at which the earliest frosts may be expected to about 10 days after the average date of first frost.

While the more deeply colored varieties increased in carotenoid pigments much more rapidly than the paler ones, both carotene and total carotenoids increased in all varieties as the season advanced; reached a maximum and then declined late in the season as climatic conditions became progressively less favorable for growth. Except for Yellow Jersey the same was true of

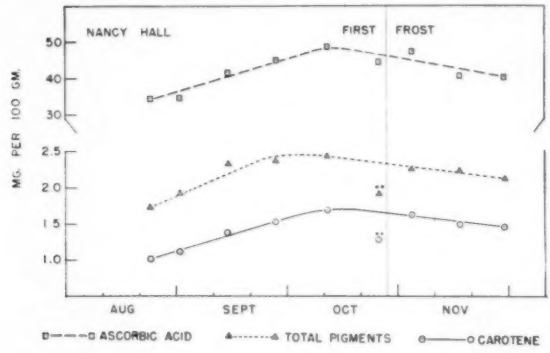

FIG. 2. Changes in the ascorbic acid, carotene and total carotenoid pigments of Nancy Hall sweetpotatoes during the harvest period.

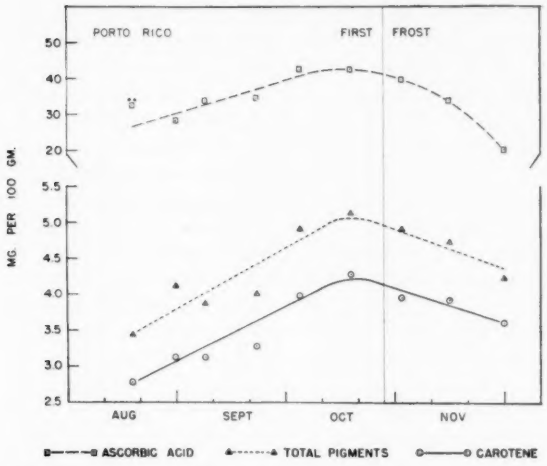

FIG. 3. Changes in the ascorbic acid, carotene and total carotenoid pigments of Porto Rico sweetpotatoes during the harvest period.

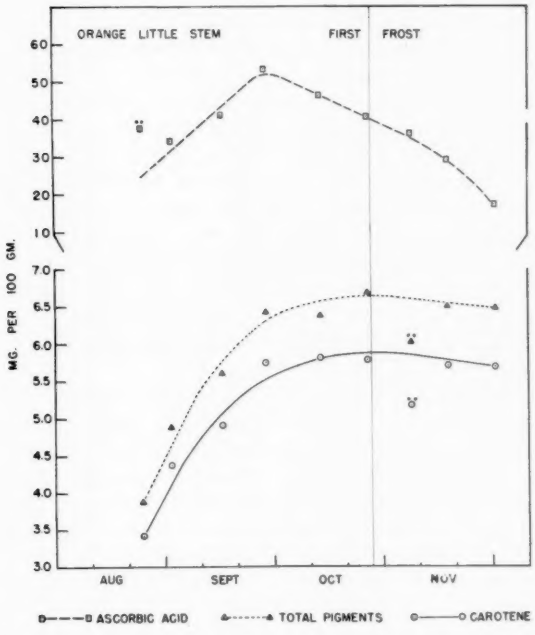

FIG. 4. Changes in the ascorbic acid, carotene and total carotenoid pigments of Orange Little Stem sweetpotatoes during the harvest period.

ascorbic acid. In this variety the ascorbic acid content at the first harvest was about the same as in the other varieties, but it failed to increase appreciably and after the fifth harvest it declined as it did in the others.

The rate of decline in ascorbic acid late in the season differed among the varieties. The roots of Yellow Jersey and the Orange Little Stem tend to grow in clusters near the surface and probably had less protection from the cold than those of the other varieties. This is indicated by approximately one half of the roots of these two varieties showing visible injury and being discarded at the last harvest. The Nancy Hall and the Porto Rico tend to be distributed more widely and deeply in the soil and showed comparatively little visible injury at the last harvest. Yet Porto Rico contained only 47% as much ascorbic acid at that time as it had earlier, while Nancy Hall contained 83% as much; Yellow Jersey contained 60% and Orange Little Stem 32% of the maximum present earlier in the season.

While the general trend of the carotene, total carotenoids and ascorbic acid contents during the harvest season thus appears to be fairly well established, there were individual fluctuations between sampling dates that we are unable to ascribe to definite causes. Some of these were statistically highly significant. They may be due to growing conditions and reflect the response of the roots to definite factors favorable, or unfavorable, to their growth and the accumulation of these constituents. Growing conditions might well be the reason for the decreases observed in ascorbic acid between the first and second harvests. But when a decrease occurs in one variety while an increase occurs in another as happened with carotenoids in Nancy Hall and Orange Little Stem at the seventh harvest the connection, if any, is more obscure; but the possibility is not eliminated. If ascorbic acid and carotene are used in the metabolism of the plant, as has been repeatedly suggested, a marked change in the metabolic rate of the roots might cause a change in these constituents also. Only one variety was harvested on each day and no data are available as to what the other varieties would have shown on those days. Nor are there any data to show that roots of different varieties respond to changes in environmental conditions at the same rate.

It may be that some of the variation is due to sampling procedure. The samples used in this study are perhaps less representative of the population than those used in the storage studies. Here several roots for the sample often came from each of a comparatively small number of plants while many more plants were harvested to make up the storage lots and the sample is likely to have been made up from a greater number of plants. Comparatively little is definitely known regarding the factors governing the development of the roots or the accumulation of ascorbic acid and carotenoids in sweetpotatoes. It is generally believed that warm nights, as well as warm sunny days are conducive to rapid growth of the roots, but few data are available as to whether accumulation or depletion of these constituents occurs simultaneously or subsequently. There is some evidence that

changes in ascorbic acid follow changes in metabolic rate and that the ascorbic acid content may even decrease temporarily while the metabolic rate is increasing. Ascorbic acid normally tends to be more concentrated in the more active tissues, yet young sprouts, mostly one inch or less in length, were found to contain far less ascorbic acid than the mother roots from which they came. Two individual samples showed 1.33 and 0.27 mg. ascorbic acid per 100 gm. of sprouts compared with 17 mg. per 100 gm. in the mother roots, although the metabolic rate in the young shoots must have been high.

To determine what effect a rapid change in metabolic rate would have on the ascorbic acid content, roots were transferred late in the storage season from 60 to 85° F at high humidity. These conditions are comparable with those during the curing process, the latter normally resulting in a rather rapid loss of ascorbic acid. The roots placed at the higher temperature sprouted readily and profusely, indicating greatly accelerated metabolic activity. Sprouts from sweetpotatoes generally appear first and most profusely near the proximal end and then in progressively decreasing numbers farther back. Different portions of the root may therefore vary in degree of metabolic activity and in the time at which changes in ascorbic acid would be evident. For this reason the roots were divided into proximal, center, and distal sections at time of sampling, each including about one third the length of the root. Each of these sections was then treated as a separate sample, the number of roots, replicates, and procedures being the same as described for whole roots. Our supply of roots for these tests was very limited and our timing did not always show an initial decrease and subsequent increase of ascorbic acid at the higher temperature. A larger supply of the Yellow Jersey variety than of the others permitted a greater number of samples to be taken and the ascorbic acid results for this variety

TABLE I

CHANGES IN THE ASCORBIC ACID CONTENT OF YELLOW JERSEY SWEETPOTATOES DURING SPROUTING FOLLOWING TRANSFER FROM 60 TO 85° F.

Treatment	Proximal section		Center section		Distal section	
	Number of sprouts	Ascorbic acid mg./100 gm.	Number of sprouts	Ascorbic acid mg./100 gm.	Number of sprouts	Ascorbic acid mg./100 gm.
Check, 60°F	0	10.6	0	12.7	0	13.0
8 days at 85°	227	11.0	59	11.2	8	10.9
15 days at 85°	433	13.2	143	14.0	31	13.9
21 days at 85°	594	11.4	189	13.2	33	13.3
28 days at 85°	....	10.2	194	11.5	51	11.5
L.S.D.*						
5%		1.02		1.31		1.16
1%		1.39		1.79		1.59

*Least significant difference.

together with the number of sprouts on the different sections when sampled, are given in table I. The sprouts were not included in the samples used for ascorbic acid. Toward the end of the test, many sprouts occurred on the proximal end in large clusters that made accurate counting difficult. On the distal section there were fewer sprouts than on the other sections, but in many cases the distal section bore fibrous roots.

The proximal section contained significantly less ascorbic acid at time of transfer than the other two sections. After eight days there was a slight, but not significant, increase in ascorbic acid in the proximal section and significant decreases in the other two sections. All sections showed an increase after 15 days and a gradual decrease thereafter as the stored reserve material was depleted. The number of sprouts showing after eight days, together with the initial decrease in ascorbic acid in the central and distal sections, followed by a later increase in all sections, supports the view that increases in ascorbic acid may follow increased metabolic activity rather than occur simultaneously. Be that as it may, the results indicate a relationship between ascorbic acid content and metabolic activity in plant tissue. It may be that the variations, the apparent departures from the normal curve during the harvest season, reflect actual changes in the metabolism of the roots, but with our present knowledge of the factors governing the growth of the roots, and the production and utilization of ascorbic acid and carotenoid pigments, we are unable to associate the variations with any specific cause or effect.

Effects of time of harvest on post-harvest behavior

The effects of time of harvest on the post-harvest behavior of carotene, total carotenoid pigments and ascorbic acid contents are shown in figures 5 to 8. From these results it is evident that pre-harvest conditions may affect the post-harvest behavior of the carotenoid pigments. With the exception of Nancy Hall, roots harvested early and at midseason increased in carotene and total pigments to a much greater degree than did those harvested late. Only in Yellow Jersey was a significant increase found in sweetpotatoes from the late harvests and this increase was relatively small. Carotene increased 39% from the late harvest compared with 366% and 473% respectively from the early and midseason harvest; total pigments increased 9% at the late harvest, significant at the 5% but not at the 1% level.

The reason for the failure of the late harvested roots to synthesize carotenoid pigments in appreciable quantities is not known. However, it is well known that sweetpotatoes are subject to chilling injury at temperatures well above freezing. The Porto Rico, Yellow Jersey, Nancy Hall and Orange Little Stem roots were harvested on November 1, 2, 3 and 7, respectively. Soil temperature records are not available, but the minimum air temperatures recorded prior to these harvests were 29° F on October 27 and 28 and 21° F on November 6. Mean temperatures for the same dates were 48, 44 and 34° F. It seems probable, therefore, that the roots were sufficiently

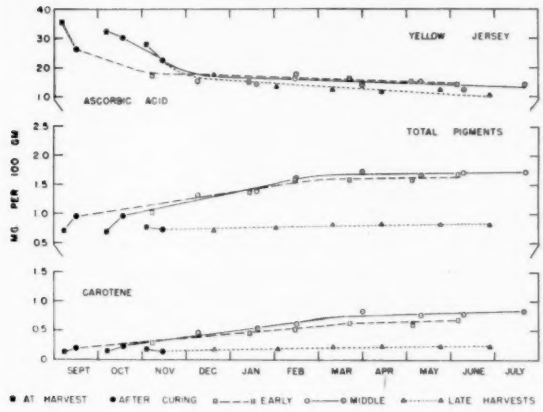
YELLOW JERSEY
ASCORBIC ACID
TOTAL PIGMENTS
CAROTENE
MG. PER 100 GM
SEPT OCT NOV DEC JAN FEB MAR APR MAY JUNE JULY
AT HARVEST AFTER CURING EARLY MIDDLE LATE HARVESTS

FIG. 5. The effect of time of harvest on the carotene, total carotenoid pigments, and ascorbic acid content of Yellow Jersey sweetpotatoes at harvest, after curing, and during storage at 60° F.

chilled before harvests to affect permanently the mechanism responsible for the synthesis of the carotenoid pigments. A few roots that failed to reach the critical temperature could account for the small increases noted in Yellow Jersey.

Another possible reason for the failure of the late harvested roots to increase in carotenoid pigments is that some substance detrimental to the roots may be passed from the frost-injured vines into the roots. This belief is responsible for the recommendation sometimes made that the vines should

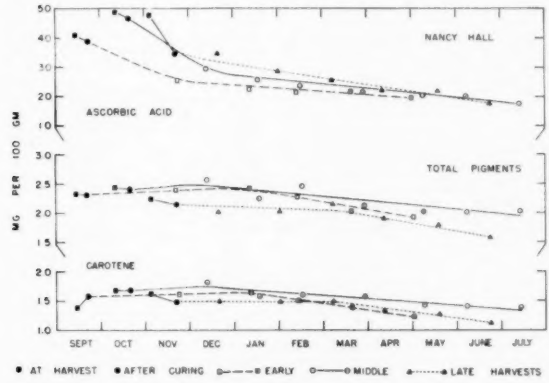
NANCY HALL
ASCORBIC ACID
TOTAL PIGMENTS
CAROTENE
MG. PER 100 GM
SEPT OCT NOV DEC JAN FEB MAR APR MAY JUNE JULY
AT HARVEST AFTER CURING EARLY MIDDLE LATE HARVESTS

FIG. 6. The effect of time of harvest on the carotene, total carotenoid pigments, and ascorbic acid content of Nancy Hall sweetpotatoes at harvest, after curing, and during storage at 60° F.

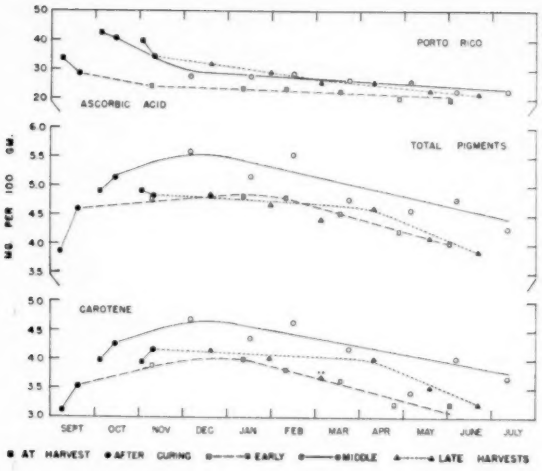
PORTO RICO
ASCORBIC ACID
TOTAL PIGMENTS
CAROTENE
MG PER 100 GM.
SEPT OCT NOV DEC JAN FEB MAR APR MAY JUNE JULY
AT HARVEST AFTER CURING EARLY MIDDLE LATE HARVESTS

FIG. 7. The effect of time of harvest on the carotene, total carotenoid pigments, and ascorbic acid content of Porto Rico sweetpotatoes at harvest, after curing, and during storage at 60° F.

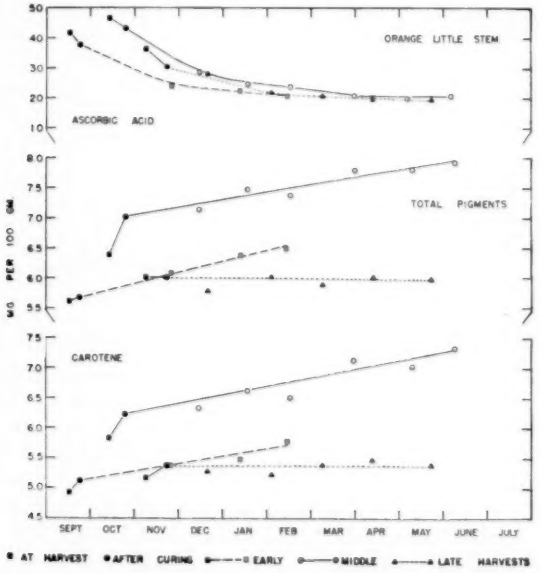
ORANGE LITTLE STEM
ASCORBIC ACID
TOTAL PIGMENTS
CAROTENE
MG PER 100 GM
SEPT OCT NOV DEC JAN FEB MAR APR MAY JUNE JULY
AT HARVEST AFTER CURING EARLY MIDDLE LATE HARVESTS

FIG. 8. The effect of time of harvest on the carotene, total carotenoid pigments, and ascorbic acid content of Orange Little Stem sweetpotatoes at harvest, after curing, and during storage at 60° F.

be severed from the roots if it is impossible to harvest the sweetpotatoes shortly after the foliage has been killed. While there seems to be few data to support this hypothesis, the opinion is still sometimes heard.

From the results with Nancy Hall harvested at different periods (fig. 6) it is again indicated that this variety is physiologically different from the other varieties studied. Carotene but not total pigments increased during curing of the early harvested lot; neither increased during curing and storage of the later harvests. The results from the early harvest confirmed our previous results which showed an increase in carotene during curing but no increase in total pigments (**3**). These facts suggest that after harvest the Nancy Hall roots are capable of synthesizing carotene from the immediate precursors, but probably are unable to synthesize the complete molecule.

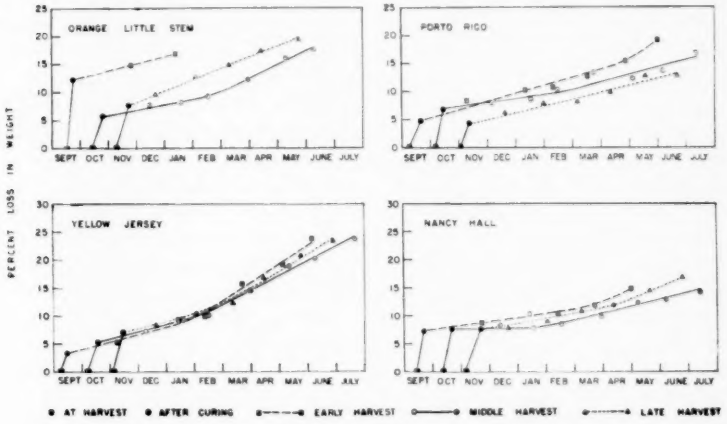

FIG. 9. The effect of time of harvest on the loss in weight of 4 varieties of sweetpotatoes during curing and in storage at 60° F.

Whether the carotene content of a particular lot increases after harvest would thus depend upon the amount of the immediate precursor present at time of harvest, which in turn probably would depend upon pre-harvest conditions. MACLEOD and UTLEY (**9**) found an increase in Nancy Hall roots during one year, but later when the variety did not show an increase in carotene during two years in succession MACLEOD (**8**) concluded that Nancy Hall roots did not increase in carotene during storage.

Rather wide differences in carotene and total carotenoid pigments are evident between sampling periods in the middle and late harvested lots of Porto Rico roots. These differences as discussed previously (**4**), are probably associated with the occurrence of internal cork with which these roots were found to be affected in late storage. The fact that the roots harvested early varied less than those harvested later gives additional support for the belief that the roots were not affected with internal cork until late in the

growing season. No indication of internal cork was observed at harvest, after curing or early in the storage period, but it was evident late in storage.

Time of harvest had relatively little effect on the behavior of ascorbic acid during storage. Rather rapid losses occurred during curing and in early storage, but time of harvest had no clear cut effect on the rate of these losses.

The per cent. loss in weight during curing and in storage is shown in figure 9. Pre-harvest environmental conditions appear to affect the loss in weight during curing, but earliness or lateness of harvest is not the primary factor governing these losses, as evidenced by the behavior of the different varieties. The rainfall preceding harvest (or soil moisture content) apparently is an important factor and appeared to bear a closer relationship to loss of weight during curing than any other factor readily evident. Earliness or lateness of harvest had no consistent effect on the moisture content of the roots either at time of harvest or during storage.

While the pigments of sweetpotatoes are composed of both carotene and non-carotene carotenoids, differences in flesh color are due primarily to differences in the carotene content. The maximum difference in non-carotene pigments between samples, treatments and varieties, amounted to about half a milligram per 100 gm. of tissue, while differences in carotene exceeded 7 mg. The varietal averages at harvest, after curing, and after storage for varying periods of time, differed in non-carotene pigments by only 0.16 mg. (table II). Three of the varieties, including the lightest and the deepest colored flesh, varied by only 0.03 mg. or less than 5% in pigments other than carotene. However, the variations within a variety, between samples, and between treatments were relatively large. This might be expected since the non-carotene fraction would probably include pigments in intermediate stages of synthesis and of degradation of the carotene molecule, as well as other carotenoid pigments. Changes in these constituents probably are not all regulated by the same factors and probably do not take place at the same rate, but reflect the sum total of all the effects of time, treatment, variety and condition of the individual roots used in the sample. The data for each variety in table II are from 10 samples taken in duplicate at harvest, and after curing. The storage lots are based on six times of sampling (unless otherwise noted) each with 10 samples taken in duplicate. The average for each variety is thus based on approximately 240 samples of five or more roots each.

In a previous paper (**4**) data were presented showing that roots stored continuously at 50° F did not increase in carotenoid pigments, while there were increases at higher temperatures. Storage at 50° F also caused roots to become more susceptible to decay organisms and thereby reduced the storage quality. The late harvested roots from Porto Rico, Yellow Jersey, and Nancy Hall varieties kept well in storage. In the late harvested Orange Little Stem a few decayed roots were present in late December and the decayed roots were discarded. Very little decay developed thereafter and the

TABLE II

THE NON-CAROTENE CAROTENOID CONTENT OF SWEETPOTATOES AS AFFECTED BY VARIETY, TIME OF HARVEST, CURING AND STORAGE.

Variety and treatment	Non-carotene pigments (mg./100 gm.)			
	Early harvest	Middle harvest	Late harvest	Average
Yellow Jersey				
At harvest	0.56	0.54	0.58	0.56
After curing	.76	.74	.58	.69
From storage	.95	.91	.58	.81
Average	.76	.73	.58	.69
Nancy Hall				
At harvest	.94	.76	.63	.78
After curing	.73	.73	.67	.71
From storage	.74*	.67	.56	.66
Average	.80	.72	.62	.72
Porto Rico				
At harvest	.75	.93	.95	.88
After curing	1.07	.88	.64	.86
From storage	.89	.85	.66	.80
Average	.90	.89	.75	.85
Orange Little Stem				
At harvest	.67	.57	.85	.70
After curing	.56	.79	.65	.67
From storage	.81**	.76	.60*	.72
Average	.68	.71	.70	.70
Average all varieties				
At harvest	.73	.70	.75	.73
After curing	.78	.78	.64	.73
From storage	.85	.80	.60	.75
Average	.79	.76	.66	....

*Five samples from storage instead of six.
**Three samples from storage instead of six.

variety was held at 60° F until late May. The other varieties were held until June without any appreciable indication of impaired keeping quality. The only ill effect noted was their failure to increase in carotenoid pigments after harvest. Whatever the cause, it appears that the failure to synthesize carotenoid pigments or a reduction in rate of synthesis in roots after harvest may be the most sensitive measure of physiological injury of sweetpotatoes yet reported.

In the present study, post-harvest losses of ascorbic acid were somewhat greater than were found previously (**5**). At that time, the maximum post-harvest losses were approximately one third of the amount at harvest, while in the present study the average amount in storage was nearer 50% of that at harvest.

Summary

Yellow Jersey, Nancy Hall, Porto Rico, and Orange Little Stem sweetpotatoes were harvested at nine times between mid-August and late November. The ascorbic acid, carotene, and total carotenoid pigments were determined at each harvest.

Carotene and total carotenoid pigments increased in the first part of the harvest period and then decreased; the maximum concentration usually occurred about the time of the usual commercial harvest for storage purposes.

The ascorbic acid content followed a pattern somewhat similar to that of the carotenoids in the Nancy Hall, Porto Rico, and Orange Little Stem varieties but the Yellow Jersey showed little change when the others were increasing. All varieties decreased in ascorbic acid late in the period.

Roots from the third (early), fifth (midseason) and seventh (late) harvests were cured, stored at 60° F and analyzed at intervals during the storage season.

The time of harvest (early, mid-season, or late) appears to be of less importance in determining the post-harvest behavior of the carotenoid pigments than do pre-harvest environmental factors.

In the Nancy Hall variety the only increase in carotene after harvest occurred during curing of roots from the early harvest. Total carotenoids did not increase after harvest in this variety. The roots of other varieties from the early and midseason harvests increased in both carotene and total pigments, but relatively little if any increases occurred in roots harvested after frost had killed the foliage.

Time of harvest had relatively little effect on the behavior of ascorbic acid during storage.

Complete or partial loss of the ability to synthesize carotenoid pigments after harvest appears to be a sensitive measure of physiological injury to sweetpotatoes.

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ABSORPTION SPECTRA OF LEAVES. I. THE VISIBLE SPECTRUM[1]

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(WITH THIRTEEN FIGURES)

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Introduction

The many and varied responses of plants to light have stimulated an interest among plant physiologists in leaf absorption spectra. The discovery by Hagenbach (**4**) of the differences in the absorption spectra of leaf extracts and leaves has led to recognition of the fact that absorption spectra of leaves are not predictable from the spectra of their extracts. More recent work by Seybold and Weissweiler (**16, 17**) has further emphasized this fact by showing that leaves which differ widely in their pigment content may show similar absorption spectra.

The measurement of leaf absorption spectra is not a simple problem. The optical difficulties involved have been discussed by several workers (**11, 14, 15, 7, 18**) who have emphasized that the problem consists essentially in measuring the diffuse light reflected from and transmitted through the leaves. If the amounts of flux incident upon, transmitted and reflected by the leaf are known, the absorption may be readily calculated from the equation $A = I - (T + R)$, where A, I, T and R refer to the flux absorbed, incident, transmitted and reflected respectively. It should be emphasized that the reflected and transmitted light is scattered in all directions by the leaf surfaces and by the tissue interfaces. It is, therefore, necessary to obtain an integrated measure of this reflected and transmitted light; and, as several workers have pointed out, the integrating sphere is well adapted for this purpose.

A study of the extent to which the reflected and transmitted light conform to the cosine law for a perfect diffusing surface has been made by Dinger (**2**), and some of his unpublished data are presented here (figs. 1 and 2). It is evident that the reflection curve for these leaves conforms closely to the cosine law, and the transmission curve conforms less closely. Both curves indicate a large amount of scattering.

Probably the most extensive study of leaf absorption spectra is that of Seybold and Weissweiler (**16, 17**). These workers used the Hardy automatic recording spectrophotometer which, although limited to the visible spectrum, is nearly ideal for this type of work. With this instrument, both reflection and transmission can be accurately and quickly measured, with

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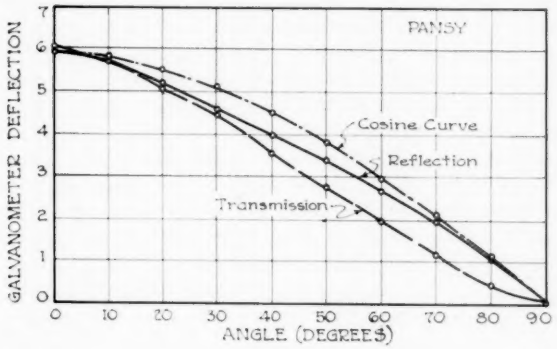
the integrating sphere principle. Absorption spectra were obtained for leaves of several species as well as for algal thalli. Normal leaves, water-infiltrated leaves and Chlorella suspensions showed an absorption maximum at about 680 mμ and a minimum at about 550 mμ. Dipping the leaves in boiling water or in ether for a few minutes shifted the absorption maximum to about 672 and 663 mμ respectively. A colloidal pigment preparation also



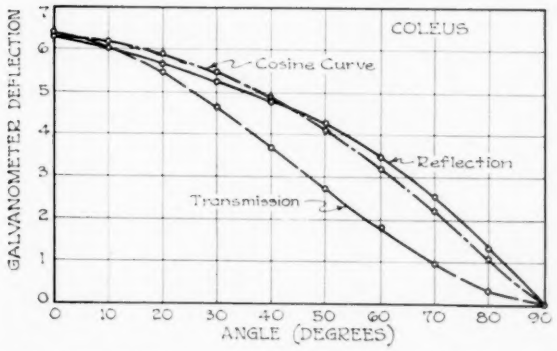


FIG. 1. (*Above*) Scattering of transmitted and reflected light by pansy leaves.

FIG. 2. (*Below*) Scattering of transmitted and reflected light by green Coleus leaves.

showed a slight shift in this absorption maximum. Comparisons between the absorption spectra of leaves grown in the shade and in the sun, and between normal and aurea leaves showed striking similarities.

More recently, RABIDEAU *et al.* (**12**) have employed the integrating sphere technique to make qualitative comparisons of absorption spectra of leaves, chloroplast suspensions and disintegrated chloroplast suspensions. With their apparatus they were able to extend the region investigated to

800 mμ. With one exception, the leaves showed an absorption maximum between 670 and 680 mμ and a minimum at 540 to 560 mμ. A leaf of Ananas gave a broad band in the red with an apparent maximum at 660 mμ. With most leaves, minor bands were evident at 600 to 620 and 640 to 660 mμ. The chloroplast and disintegrated chloroplast suspensions gave absorption spectra qualitatively similar to those of the leaves, but the bands were more sharply defined.

The present investigation was made with three purposes in mind. The first was to make a study of both the reflection and absorption characteristics of leaves and the effects of unusual leaf surfaces and of differences in leaf color. The second was to determine the effects on absorption spectra of dipping the leaves in boiling water and in ether, and of infiltrating the leaves with water. Some data relative to the effects of such treatments have already been reported by SEYBOLD and WEISSWEILER (**16, 17**) but have not as yet been confirmed by other investigators. The third purpose was to make a comparative study of the spectra of leaves, leaf extracts and chloroplast and disintegrated chloroplast suspensions. The extracts and suspensions were to be of such concentration that, when examined in a 1-cm. absorption cell, the quantity of pigment per unit cross-sectional area of light beam would be equal to that obtained with the leaf sample. In this manner the effect on absorption spectra of other variables than pigment quantity could be ascertained.

Materials and methods

The reflection data shown in figures 5 and 6 were obtained by means of a Razek-Mulder recording spectrophotometer. This instrument utilizes a diffuse light source, and the reflection normal to the leaf surface is measured by a phototube. The output from the phototube is fed to a galvanometer, and the galvanometer deflection is amplified optically and recorded photographically. The transmission data in figure 6 were obtained by clamping the leaf to a Weston photocell and exposing the leaf to the beam from a monochromator. The photocell output was fed to a galvanometer and the deflection read from a scale. Incident intensity was obtained by reading the deflection with the leaf removed from the front of the photocell. The absorption percentages were obtained by subtracting the sum of the reflection and transmission percentages from 100.

The data shown in the other figures were obtained with a spectrophotometer which was constructed specially for this work. The light source consisted of a projection lantern equipped with a 500 watt lamp. The monochromator was a single-prism instrument fitted with quartz optics. An integrating sphere, 25.5 cm. in diameter, was attached directly to the exit slit housing of the monochromator and held in place by a clamp and flange. The inner surface of the sphere was finished with a paint described by WALSH (**20**) which gives a white matt surface as required in sphere photometry.

The leaf specimen was held in the center of the sphere by a rectangular holder which was pivoted to permit swinging the leaf out of the light beam

when desired. The sample was trimmed to fit the holder and always included the midrib or a primary vein which fitted into a slot at one side of the holder and projected into a small cup of water. By this means the tendency of the sample to dry out while the measurements were being taken was greatly decreased.

An RCA type 1P-22 photomultiplier tube was used as the detector. It was mounted in the bottom of the sphere in such a manner that only reflected light from the sphere walls could strike the photocathode. The voltage supply for the tube consisted of a stabilized electronic power supply which provided a potential of from 900 to 1000 volts DC between the cathode and ninth dynode, and a separate 45 volt battery for the last stage between the ninth dynode and the anode. A complete description of this apparatus has been given by Moss (**9**). The output was read directly by means of a microammeter.

The present investigation was confined to the visible spectrum. The slits were adjusted to a calculated band width of 10 mμ throughout the spectrum, and readings were made every 10 mμ. Duplicate or triplicate determinations were made for each of the species, treatments or preparations, and either a mean value or a representative curve was chosen to be presented here. Three measurements were necessary at each wavelength setting to obtain the leaf absorption spectra. A reading with the sample in the sphere but out of the light beam gave a measure of the incident flux. A reading with the sample in the light beam gave a measure of the combined transmitted and reflected fluxes. The amount of flux reflected was measured by taking a reading with the sample in the light beam but with a metal clip directly behind it. This clip was painted a dull black on the side facing the leaf and white on the other side. Absorption was calculated from the equation $A = I - (T + R)$, where A, I, T and R refer to the flux absorbed, incident, transmitted and reflected respectively.

The absorption spectra of the extracts were measured in the same apparatus by placing first the solvent, then the extract, in a 1 cm. Beckman absorption cell which was mounted between the exit slit of the monochromator and the sphere. The spectrum was scanned three times with the solvent in the cell, then three times with the extract in the cell. Readings were taken every 10 mμ, and mean values computed. The absorption was calculated by taking the difference between the transmission of extract and solvent and expressing this difference as a percentage of the transmission shown by the solvent. This method involves the customary assumption that the refractive indices of solvent and extract are essentially the same. A similar procedure was followed in measuring the absorption spectra of the chloroplast and disintegrated chloroplast suspensions, except that the cell containing the suspending medium or suspension was mounted in the leaf holder in the center of the sphere. A correction for reflection from the cell and suspended chloroplasts was made by blocking off the transmitted light with a piece of dull black paper. Several tests were made to determine the accuracy which could be expected from the apparatus. All indicated that

the amount of scattered light was negligible, and that instrumental errors were within the limits required. Transmission curves obtained with the apparatus described above and with a Beckman spectrophotometer checked within 2%, except in steep portions of the curves where the discrepancy was attributable to band width differences.

Most of the plant material used was grown in the greenhouse. Care was taken to avoid drying or deterioration of the material prior to the determinations. Unless otherwise stated, the sample was mounted in the sphere in such a manner that the light was incident on the upper surface of the leaf. The leaf extracts were prepared by essentially the same method as that used by SEYBOLD and WEISSWEILER (**16, 17**). Twenty-five square centimeters of leaf tissue were extracted with methanol and the extract made up to a volume of 25 ml. The extracts were stored in the dark at zero degrees centigrade, and analyzed as soon after the extraction as feasible.

The chloroplast suspensions were prepared by a modification of the method reported by GORHAM and CLENDENNING (**3**). The filtered brei was centrifuged for 10 minutes at 300 to 350 g. to eliminate the large particles, and the chloroplasts were thrown down by centrifuging 10 minutes at 2000 g. and resuspended in 0.5 M sucrose. The disintegrated chloroplast suspensions were prepared by grinding the leaf laminae for 10 minutes in a mixture of ice and distilled water with the Waring Blendor. The material thrown down by centrifuging 10 minutes at 4000 g. was discarded, and that which was thrown down at 20,000 g. was resuspended in distilled water and kept for analysis. The suspensions were made up to a volume such that they would have the same quantity of pigments per unit volume as the extracts. This was done by centrifuging the solid material from an aliquot of the suspensions, extracting this material with methanol, and comparing the extract with the equal-area leaf extract using a Duboscq colorimeter. From this comparison, the proper dilution factor for the suspension was calculated. As a result, the leaves, leaf extracts, chloroplast suspensions and disintegrated chloroplast suspensions were all comparable as to quantity of pigment per unit cross-sectional area of light beam.

Experimental results

Absorption and reflection spectra for several species were obtained; but, because of their similarity, not all of them are presented here. The results obtained with six species are shown in figure 3. Bean, spinach, Swiss chard and tobacco were chosen as representative of species having relatively thin leaves with no unusual surface characteristics. A considerable uniformity in their absorption and reflection spectra will be noted, with the exception of the high reflection and low absorption shown by tobacco in the neighborhood of 550 mμ. The absorption minimum at 550 mμ, the points of inflection at about 600 and 630 mμ, the maximum in the neighborhood of 680 mμ and the sharp drop beyond 680 mμ are typical of most of the species examined. Much less detail is shown in the absorption spectra at the shorter wave-

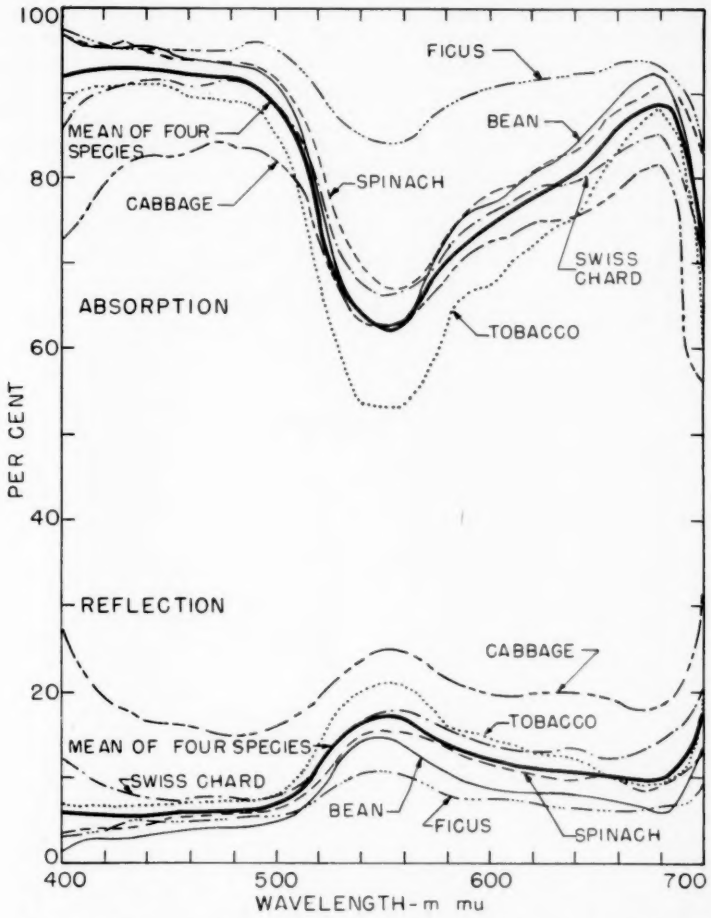


FIG. 3. Absorption and reflection of light by leaves of six species, together with smoothed average curves for bean, spinach, Swiss chard, and tobacco. Reflection of green light (550 mμ) averaged 17% and absorption 62%.

lengths. The heavy lines represent the mean values of the absorption and reflection spectra of the four species. It should be noted that the curves of reflection and absorption are roughly complementary, although less detail is shown by the reflection curve.

Ficus was chosen to represent the thick, heavily pigmented type of leaf. As compared with the more normal type, it showed high absorption across the entire visible spectrum, with the absorption bands less clearly defined

and with less difference between the absorption at 550 and 680 mμ. The absorption peak in the red was spread out between 660 and 680 mμ. The reflection was correspondingly low and showed relatively little variation.

The effect on absorption and reflection spectra produced by the presence of a highly reflecting material on the leaf surface is shown by the curves for cabbage. Reflection was 8 to 28% higher than the mean curve for the four normal species. Absorption was low, though not so low as tobacco in the region of 500 to 630 mμ. Part of this difference can be attributed to the fact that the reflection curve for tobacco showed greater variation in this region.

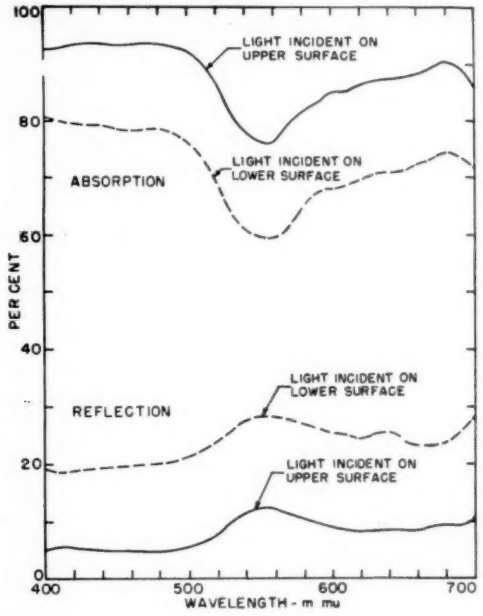


FIG. 4. Absorption and reflection of light incident on the upper, dark green, or lower, white, surface of white poplar leaves.

The effect of surface character is shown even more strikingly by the curves for white poplar in figure 4. These were obtained with the light incident first upon the upper, then the lower surface of the leaf. The difference in the absorption curves can be accounted for almost quantitatively by the difference in reflection. It should be noted that the absorption curve for the light incident upon the upper surface follows the general pattern set by the curves in figure 3, but is intermediate between that of such species as spinach and that of Ficus.

The relationship between leaf color and reflection spectra is shown in figure 5. It is interesting that yellow and orange leaves show greater reflection in the green than does the green leaf. However, the high reflection

throughout the yellow and red accounts for their characteristic colors. Reflection in the blue is nearly the same for leaves differing greatly in their apparent color. It should be remembered that reflection from leaves is made up of two components. One is the reflection occurring at the first air-cuticle interface, which probably is fairly uniform throughout the spectrum. The other is the reflection occurring at interfaces within the leaf. This second component of reflection is subjected to selective absorption by the materials through which the light must pass, and accounts largely for the spectral character of the reflection curves. This point is supported by the

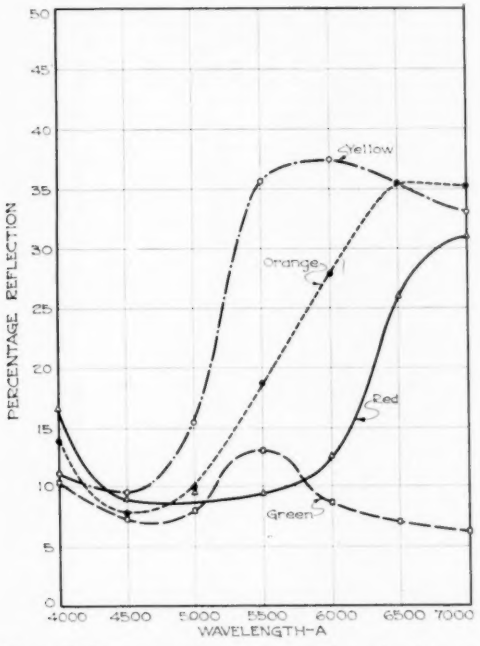


FIG. 5. Reflection spectra of green and fall colored leaves.

curves for infiltrated leaves in figures 10 and 11, which show that infiltration reduces the internal reflection and thereby tends to flatten the curve.

Figure 6 shows the reflection, transmission and absorption for yellow leaves. It will be noted that the transmission and reflection curves are similar and of the same order of magnitude. Both are complementary to the absorption curve.

ABSORPTION AND REFLECTION SPECTRA OF TREATED LEAVES

The effect of boiling upon the absorption spectra of leaves is shown in figures 7 and 8 for bean and Kalanchoe. The spectrum of the crude methanol extract is included in figure 7 for comparison. The relatively high

concentration of acids in Kalanchoe leaves makes the preparation of pigment extracts difficult. The effect of boiling the bean leaf was principally to decrease its absorption in the region beyond 480 mμ and to shift the position of the absorption peaks toward the blue. There was a significant decrease in reflection beyond 520 mμ. The decrease in absorption and reflection can be attributed to a partial filling of the air spaces in the leaf by water. The shift in the spectrum was most noticeable in the far red, and caused the peak to move to a position intermediate between that for the normal leaf and the extract.

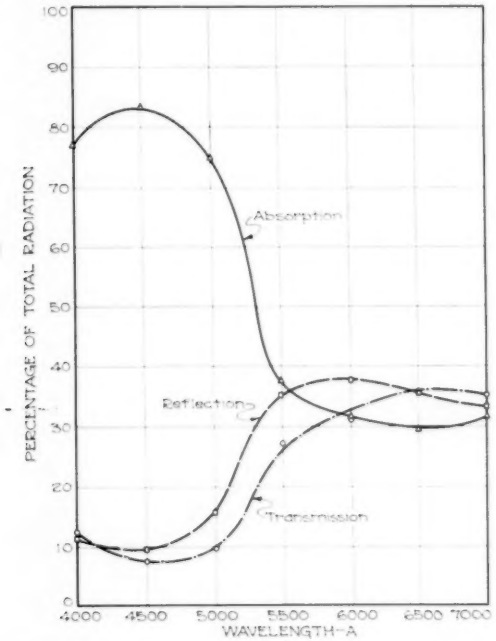


FIG. 6. Average transmission, reflection and absorption spectra for a number of yellow leaves.

The spectrum of the boiled Kalanchoe leaf showed a different pattern, although the blue shift in the peak at 680 mμ was evident. In the absorption curve for bean there were suggestions of absorption bands at 500, 580 and 620 mμ. In the curve for Kalanchoe there was no suggestion of a band at 480 mμ; a new band was found at 540 mμ, and one at 620 mμ. Even more striking were the shift in the absorption minimum from 550 to 585 mμ and the increased reflection in the red. It would be difficult to arrive at an adequate explanation for these differences without careful chemical and spectrophotometric work. A probable explanation may be found in the high acid content which leaves of succulents often contain. With heating, this

acid could lead to pigment degradation, as shown by the development of brown coloring in heated leaves.

The effects of dipping the leaves in ether as well as those resulting from dipping in boiling water are shown in figures 9, 10 and 11. The effect of the boiling was much the same for spinach and tobacco as for bean, resulting principally in an overall reduction in reflection and absorption at the longer wavelengths, and a shift which was particularly noticeable in the

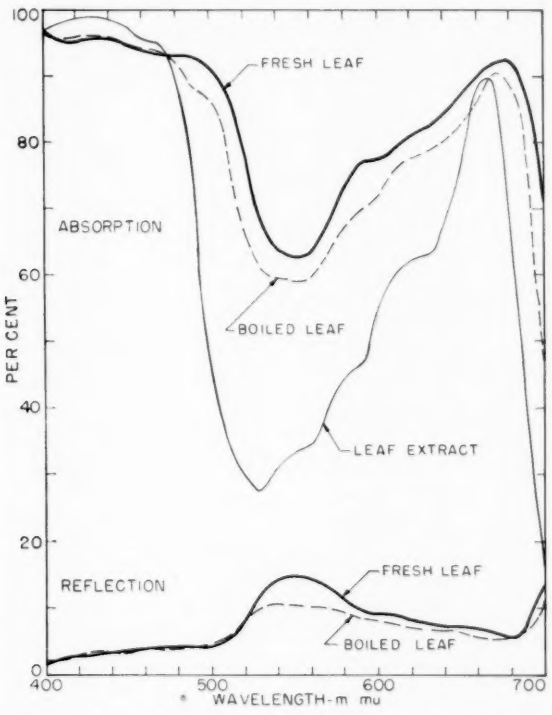


FIG. 7. Transmission and reflection spectra for bean leaves and a methanol extract containing an equal quantity of pigments.

position of the absorption peak in the red. The curves obtained for a Swiss chard leaf dipped in boiling water resemble those obtained for Kalanchoe. The same absorption peaks at 540 and 620 mμ and the shift in the absorption minimum may be found.

There was also a lack of uniformity in the results obtained by dipping the leaves in ether. With spinach, both the absorption and reflection curves were shifted toward shorter wavelengths. The shift in the position of the red absorption maximum was greater than was obtained by dipping the leaf in boiling water, but the peak was not shifted so far as that of the ex-

tract. Although the amount of reflection was only slightly altered, the absorption was markedly decreased. The curves for Swiss chard and tobacco present a different aspect. With Swiss chard, the reflection maximum, which was at about 550 mμ in the fresh leaf, had been nearly eliminated by the ether dipping, and with tobacco the maximum had been lowered greatly. This reduction was reflected partly in the increase in absorption throughout the region, but it should be noted that the increase in absorption was approximately double the reduction in reflection. With both species, the posi-

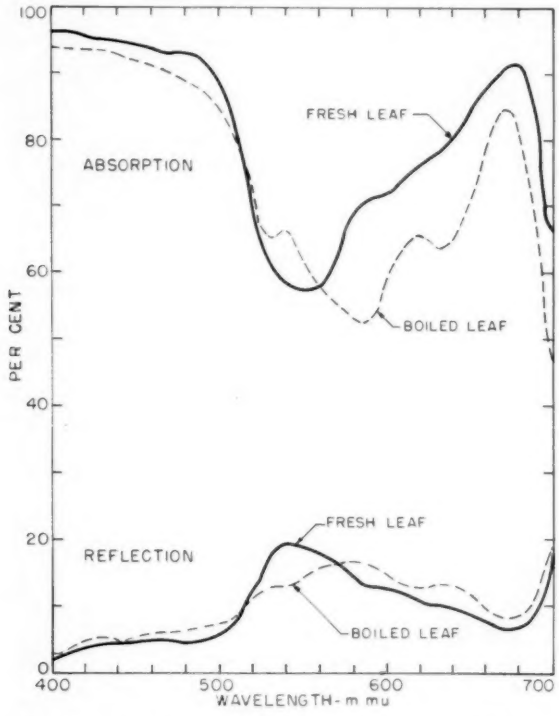


FIG. 8. Spectra of live and freshly boiled leaves of Kalanchoe.

tion of the red absorption maximum coincided with that obtained by dipping the leaves in boiling water. A possible explanation for these differences may be found by considering the relative thickness and permeability of the leaves and the length of time they were immersed in the ether, since these factors would affect the extent to which the pigments were extracted from the chloroplasts and the extract diffused throughout the leaf tissue. In no case was there any observable removal of pigment from the leaf.

The curves obtained from leaves infiltrated with water are typical of those obtained with several species. The effect was to reduce the internal

reflection at interfaces within the leaf tissue and thus lower both the reflection and absorption. The position of the principal absorption bands remained unchanged.

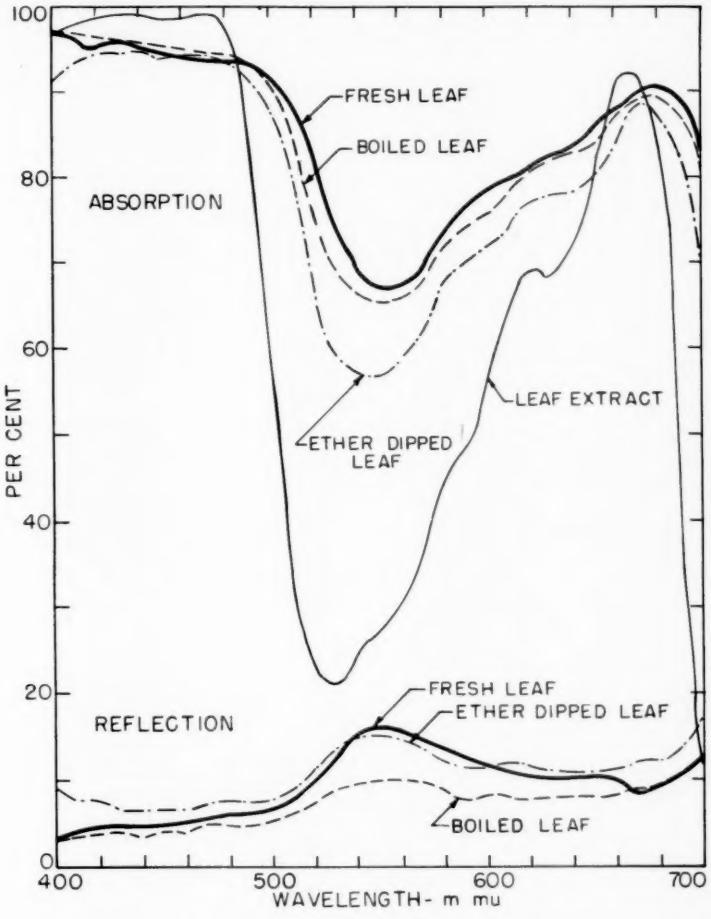


FIG. 9. Leaf and extract spectra for spinach.

ABSORPTION SPECTRA OF CHLOROPLAST AND DISINTEGRATED CHLOROPLAST SUSPENSIONS

The results of a series of experiments designed to compare the absorption spectra of fresh leaves with those of chloroplast and distintegrated chloroplast suspensions of such concentration that quantity of pigment would not be a factor are summarized in figures 12 and 13. There was a progressive

narrowing of the principal absorption bands from the fresh leaf to the chloroplast and distintegrated chloroplast suspensions, but the position of the maxima and minima remained essentially unchanged. In the curves for spinach the absorption in the blue and in the neighborhood of 680 mμ was

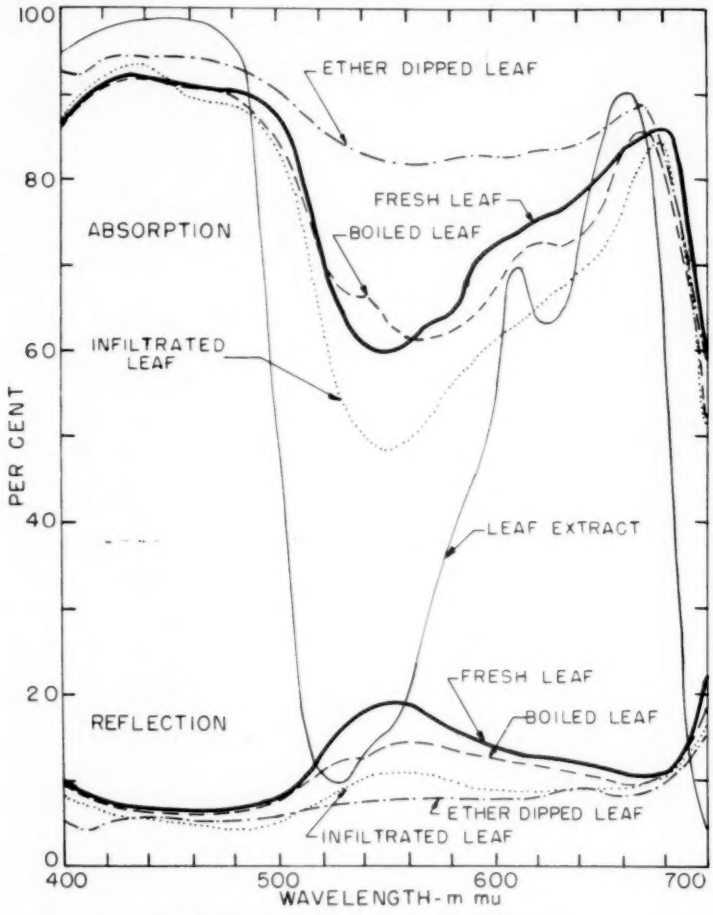


FIG. 10. Leaf and extract spectra for Swiss chard.

higher for the distintegrated chloroplast than for the chloroplast suspension, and the reverse was true in the curves for Swiss chard. A small error in the dilution factor for one or more of the suspensions could account for this difference.

Because of the many indications that the pigments in the chloroplasts may be in a colloidal state, the absorption curve for a colloidal suspension

of the crude pigment extract from Swiss chard is presented in figure 13. Several differences between this curve and that obtained for the fresh leaf will be noted. There was a shift of nearly 40 mμ in the position of the absorption minimum for the colloidal suspension, and a shift of at least 5 mμ

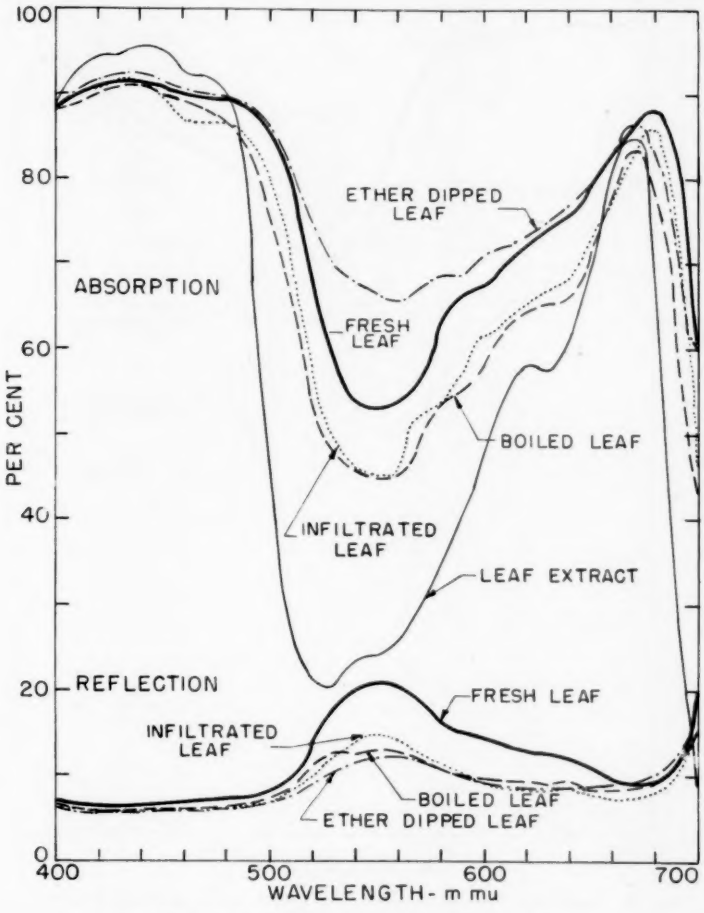


FIG. 11. Leaf and extract spectra for tobacco.

in the position of the red maximum. The latter shift has been noted by many other authors.

Discussion

The success of any spectrophotometric study is dependent to a large degree upon the accuracy of the apparatus used. The instrumental problem

is complicated by the optical nature of the material to be investigated. Leaves contain a variety of pigments whose physical state in the living leaf remains unknown. A considerable portion of the incident light is reflected

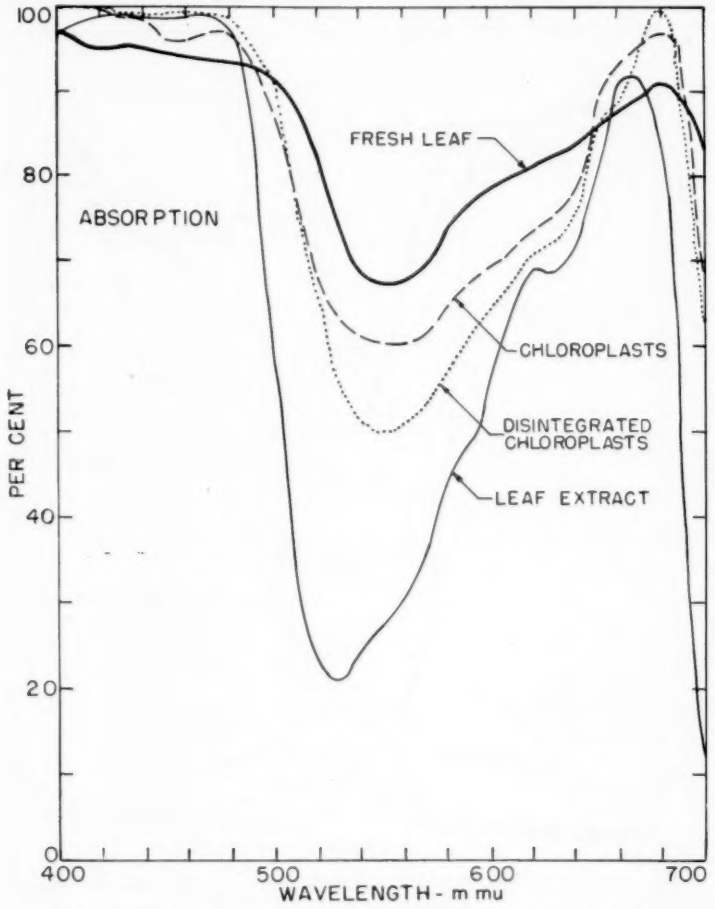


FIG. 12. Absorption spectra of equivalent quantities of pigments from spinach leaves in four physical states. Note that the high absorption of green light and the red band at 680 mμ are characteristic of all the curves except that for the methanol extract.

from the external surfaces, and another portion which is internally reflected is modified by the selective action of the pigments through which it passes. This reflected light is neither perfectly spectral nor diffuse in character, and the degree to which it approaches the cosine law for diffuse reflectance varies

between species and, to some degree, within a single species. The transmitted light is scattered also. The numerous air spaces and interfaces in the leaf tissue cause internal reflection which lengthens the effective light path within the leaf. As a result of all these factors, the absorption spec-

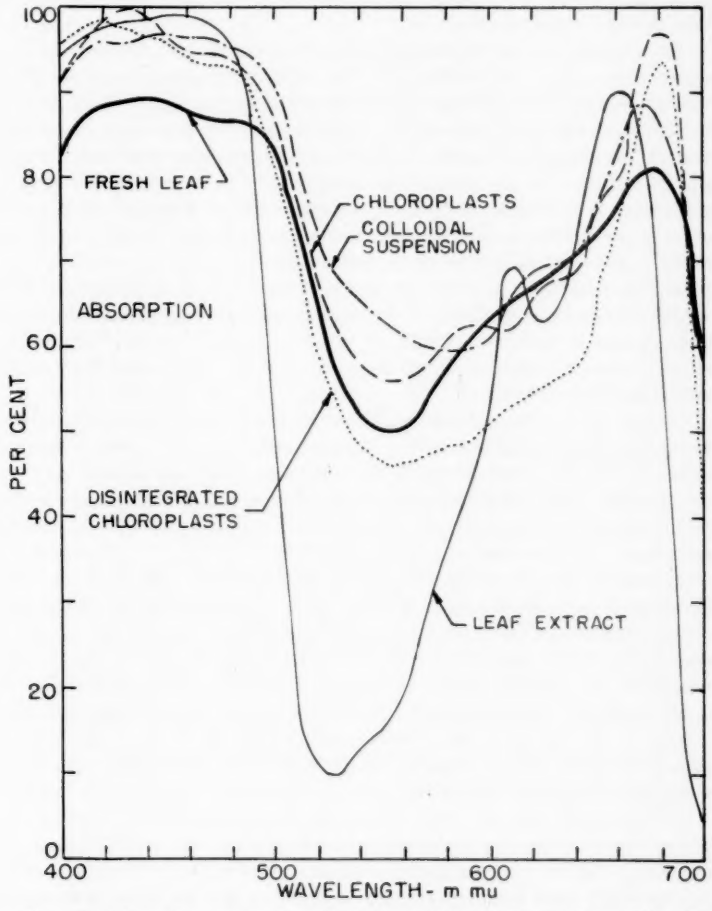


FIG. 13. Absorption spectra of equivalent quantities of pigments from Swiss chard leaves in five physical states.

trum of the leaf as a whole can be obtained only by an integrated measurement of the scattered light reflected from and transmitted through the leaf. The absorption spectrum of the pigments within the leaf is still more difficult to obtain, and will be discussed later. The selection of the com-

ponent parts of the apparatus used in the present work was influenced largely by their availability. The major choice was in the type of detector and its accompanying circuit. The selection of a photomultiplier tube proved to be a wise one from the standpoint of simplicity, economy and linearity of response. It is believed that the apparatus used was entirely adequate for the purpose.

Figure 3 shows that the absorption curves for most of the species have a maximum at or close to 680 mμ. The corresponding maximum for Ficus was not clearly defined but was between 660 and 680 mμ. The absence of a distinct absorption maximum, or an apparent shift in its position toward shorter wavelengths, is typical of thick or heavily pigmented leaves with a glossy surface. If the percentage transmission is computed for a dark, glossy leaf such as that of Ficus in the region 650 to 690 mμ, values of 0.5 to 3.0% are obtained. The shape of the absorption curve is then clearly dependent upon the reflection curve and does not correspond exactly to the absorption characteristics of the leaf. If such a leaf is infiltrated with water, the internal reflection will be reduced and a more pronounced absorption peak at or near 680 mμ will be found. This relationship between the reflection and absorption curves must be kept in mind when interpreting leaf absorption spectra.

Several investigators have attempted to study the absorption spectrum of the pigments in the leaf but no critical analysis of the problem has yet appeared. Brown and Escombe (**1**), Seybold (**15**) and others have attempted to measure light absorption by white and green portions of varigated leaves. However, it was pointed out by Willstätter and Stoll (**21**) and again by Schanderl and Kaempfert (**13**) that such a procedure is invalid because it neglects the effect of internal reflection and implies that all the pigment is concentrated in a layer along the lower surface of the leaf. Other investigators have used water-infiltrated leaves and have compared the absorption spectra before and after extracting the pigments with alcohol. This procedure is objectionable in other respects. A partial solution to the problem can be obtained by using the white and green portions of a varigated leaf and infiltrating both portions with water to reduce the internal reflection. Unfortunately, it is difficult to find a pigment-free portion of leaf tissue large enough to fill the light beam of the instrument employed.

Seybold and Weissweiler (**17**) have attached considerable importance to the relative absorption in the three broad regions 400 to 500, 500 to 600 and 600 to 700 mμ. The absorption percentages obtained by these workers for *Sambucus niger* for these regions were 90, 74 and 83, and the ratios of absorption were respectively 108 : 89 : 100. An estimate of the absorption percentages for these three regions was made from the mean absorption curve for four species in figure 3. The resulting percentages were 92, 71 and 84; and the ratios were 110 : 85 : 100. The average absorption for the entire visible spectrum was estimated to be 82% and the reflection 10%.

Seybold and Weissweiler also obtained curves comparing the absorption and reflection by white poplar leaves when the light was incident on the

upper and on the lower surfaces. Their data show a difference of about 30% in both reflection and absorption. Figure 4 shows that the difference obtained in the present investigation was about 15%. This discrepancy can probably be attributed to the use of a young leaf in the present work on which the white hairs characteristic of the lower surface were not fully developed.

The discovery by HAGENBACH (**4**) of the shift in the absorption curve of the extracted pigments, and SORBY's discovery (**19**) of the effect on the absorption curves of leaves produced by dipping them in boiling water find confirmation in the data presented here. The effect produced by dipping the leaves in ether was investigated by SEYBOLD and WEISSWEILER (**16**), and their results are to a large degree substantiated here. The curves for spinach (fig. 9) contain a strong suggestion that the spectra of the boiled leaf and ether-dipped leaf are intermediate between the spectrum shown by the fresh leaf and that shown by the methanol extract. That dipping a leaf in ether should result in an absorption spectrum similar to that of an extract is not surprising, for the effect seems to be a partial extraction with the ether extract diffused throughout the leaf tissue. The same bands which appear in the spectrum of the extract were found in the spectra of the ether-dipped and boiled leaves, and suggested in the spectrum of the fresh leaf.

The absorption curves obtained with treated leaves of other species do not conform so well to the progressive transition found for the treated leaves of spinach. The absorption curve for the boiled Kalanchoe and Swiss chard leaves (figs. 8 and 10) were clearly out of line with this suggestion. They are most easily explained as resulting from degradative changes caused by leaf acids. In both species the boiled leaves had the brown color associated with phaeophytin. The effect of dipping the leaves in ether appeared to vary with the length of treatment. With prolonged treatment there was a marked reduction in reflection and a corresponding increase in absorption. With this increase in absorption, the bands became indistinct and a more uniform curve resulted, as shown by the curves for Swiss chard in figure 10 and to a less extent for tobacco in figure 11. The leaves of these species were given a much longer treatment than that given the spinach leaf. The absorption curve for the boiled tobacco leaf was similar to that for the spinach. However, the curve was intermediate in position between that of the fresh leaf and that of the extract.

SORBY (**19**) explained the boiling effect on leaf spectra by stating that the pigments were initially present in the free state, and, on heating, became dissolved in melted fatty material. WILLSTÄTTER and STOLL (**21**) arrived at essentially the same conclusion on the basis of the observation that the spectrum of the boiled leaf was like that of a solution of chlorophyll in phytol. It may be argued that boiling is an extremely harsh treatment for so labile a system as that of the plant pigments in the living leaf. However, NOACK (**10**) showed that distinct changes could be produced by temperatures much lower than that of boiling water. He prepared a colloidal dispersion of leaf material and showed that the natural fluorescence was mark-

edly decreased by heating to 70° for a few seconds. Longer heating caused a reappearance of the fluorescence. These reactions were interpreted as indicating that the chlorophylls are initially bound to protein and that this bond was broken by heating, leaving the chlorophylls in a colloidal state. Further heating caused them to become dissolved in the melted lipoids. MESTRE (**6**) investigated the effect of heating leaves and found a time-temperature relationship for the shift in the absorption spectrum which closely paralleled that for albumin coagulation. A similar effect was obtained for chloroplast suspensions by HAGENE and GOAS (**5**). Both of these latter investigations tend to support the conclusions of Noack.

The data presented here are consistent with Noack's hypothesis that the initial effect of heating is the denaturation of protein with rupture of the chlorophyll-protein bond, followed by formation of a solution of chlorophyll in melted lipoids. However, the data do not exclude other possible explanations, and the existence of an intermediate colloidal state, as proposed by Noack, remains unproved.

An attempt to obtain some data relative to the problem of determining the amount of absorption which can be attributed to the pigments in a leaf as distinct from the leaf as a whole has been made by determining the absorption spectra of chloroplast and disintegrated chloroplast suspensions. These suspensions were of such a concentration that the amount of pigment contained in an aliquot of suspension placed in a 1 cm. absorption cell was the same as that in a portion of leaf of equivalent area. The results are presented in figures 12 and 13, with the absorption curves for fresh leaves and leaf extracts included for comparison. Some discrepancy is found between the curves in the two figures which is probably due to errors in computing the proper dilution factors. However, the data indicate that as progressively smaller particle sizes were used there was a progressive reduction in absorption throughout the central portion of the visible spectrum and a sharpening of the absorption maxima and minima. Reflection from the disintegrated chloroplast particles was detectable only in the region of 500 to 600 mμ. Reflection from whole chloroplasts was appreciable throughout a broader region. The effect of reducing the particle size was partly a reduction of reflection which, in turn, tended to raise the absorption maxima and lower the minima. The same absorption bands were found in the suspensions as in the fresh leaf, and no shift in their position had occurred.

MILNER *et al.* (**8**) have recently described the preparation of very fine suspensions of chloroplast material. These suspensions, when activated by the addition of neutral salts in the presence of methanol, show an appreciable amount of the photochemical activity associated with the original chloroplasts as measured by the Hill reaction. Furthermore, successive precipitation and resuspension produces photochemically active material with an essentially constant chlorophyll/protein ratio. Absorption spectra for these suspensions were not given. Since the activity was found to be dependent upon the degree of aggregation, it would be interesting to know

what effect, if any, the state of aggregation had upon the absorption spectra. A correlation between the effect of various treatments on the photochemical activity and on the absorption spectra would also be of interest. From their work it appears that the fundamental photochemical unit must involve an association of both lipoid material and protein with the pigments.

Summary

Absorption and reflection spectra for leaves of four dicot species possessed certain features in common. There was an absorption maximum in the red at 680 mμ and a minimum in the green at 550 mμ, with points of inflection suggestive of minor absorption bands at about 600 and 640 mμ. Absorption was high throughout the blue but no distinct peak was present.

Mean absorption and reflection curves were obtained from the data for these four species. From this mean absorption curve, average values of absorption for the three regions 400 to 500, 500 to 600 and 600 to 700 mμ of 92, 71 and 84% respectively were obtained. For the region 400 to 700 mμ, the absorption was 82% and the reflection 10%.

Curves obtained for Ficus show the reduced reflection, increased absorption and general flattening characteristic of the absorption and reflection spectra of thick leaves. The white, tomentose, lower surface of a white poplar leaf resulted in an increase in reflection of about 15% and a corresponding decrease in absorption throughout the spectrum when the light was incident on the lower surface. Yellow, orange and red leaves showed about the same reflection in the blue but a much higher reflection in the red, as compared with a green leaf. The yellow and orange leaves reflected much more of the green and yellow light than did the green leaf. Transmission and reflection spectra for yellow leaves were similar, and both were complementary to the absorption spectrum.

Dipping leaves in boiling water or in ether for a few minutes shifted their absorption spectra toward the blue and sharpened the absorption bands. The change was more pronounced when the leaves were dipped in ether than when they were boiled. The boiled leaves showed a reduced absorption throughout the central portion of the spectrum. Those dipped in ether showed either an increased or decreased absorption throughout this region, depending primarily on the length of treatment. Leaves infiltrated with water showed no shift in their absorption spectra, but rather a decrease in absorption throughout most of the visible region and a sharpening of the band at 680 mμ.

Some data relative, to the absorption spectrum of the pigment complex freed from interference by the leaf structure were obtained by comparing the absorption spectra of leaves and leaf extracts with those of chloroplast and disintegrated chloroplast suspensions. These suspensions and extracts were so prepared that, when placed in a 1 cm. absorption cell, the quantity of pigment per unit cross-sectional area of light beam was the same as that obtained when using a sample of leaf tissue. The maxima and minima for

the suspensions agreed in position with those for the fresh leaf, but were more sharply defined. The effect of progressively smaller particle size in reducing the multiple reflection and thereby the absorption, particularly in the green, was also evident.

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THE ROLE OF ORTHO-SUBSTITUTION IN THE SYNTHETIC AUXINS

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(WITH THREE FIGURES)

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Introduction

The structure of molecules active as auxins has been investigated extensively, and presents one of the most attractive of all problems of the relation between a chemical structure and a biological activity. The structures are relatively simple, the compounds generally stable and the tests reproducible and quantitative. The results and conclusions to date were reviewed in 1949 (**13**). The general rules for the structural requirements of active molecules which were given more than 14 years ago (**5**) have been slightly modified and restated to fit certain new cases (**16, 17**), but basically new departures in principle have not appeared until quite recently. There are now, however, two important new points to consider. One of these is the proposal of MUIR and co-workers (**3, 9, 10**) that in order for a substance to have auxin activity, at least one of the positions on the aromatic ring ortho to the acidic side chain must be unsubstituted. This proposal stemmed from the inactivity (or very low activity) of 2,4,6-trimethylphenylbutyric acid, 4,7-dichloro-2-methylindoleacetic acid, and particularly the 2,4,6-trichloro-, tribromo- and trimethyl-derivatives of phenoxyacetic acid. These authors used the elongation of Avena coleoptile sections as a test. It has been pointed out earlier (**15**) that the Avena coleoptile is more selective than the pea stem. However, the present paper shows that also in pea stems these latter three compounds are of very low activity.

The second new principle is the activity of certain benzoic acid derivatives, reported (**1**) and confirmed (**10**) for growth of Avena sections, and which is also confirmed herein for the pea test. Earlier suggestions of activity in this series, based on different tests, had been made for 2-bromo-3-nitrobenzoic acid and some other derivatives (**19**); but activity in the pea test had not been found (**18**). It was suggested that these compounds did not have true auxin activity (**18**). The fact that true auxin activity is present in benzoic acid derivatives means that the rule that the acid group must be separated from the ring by at least one atom (**5**), though valid in the large majority of cases (**13**), is subject to important exceptions. The same is true for the suggested explanation of this requirement, namely that the acid group must be held out of the plane of the ring (**16**).

In the present study a number of other compounds relating to the above two points have been tested, and it is shown that the role of ortho-substitution is less simple than it appeared at first. Some of the facts are consistent

with another view, namely that a pair of para positions should be unsubstituted (**8**). However, neither of these views is completely satisfactory. It is believed, nevertheless, that all the facts taken together very strongly indicate chemical reaction between the benzene ring and its substrate.

Methods

The pea test was carried out in the usual manner (**14**). After soaking for six hours the peas were germinated between moist filter papers for 36 hours and planted in Lucite holders. They received occasional, very weak, red light after planting. They were grown at 25° C in water and used at an age of about seven and one half days. Third internodes were used from only those plants in which the fourth internode did not reach 2 mm. in length. After slitting, these internodes were washed three to four hours in distilled water, then floated in the test solution. All operations were carried out in red light (> 5700 Å) and the curvatures allowed to develop in complete darkness. Solutions were brought to pH 5.5 with NaOH but otherwise

TABLE I

ACTIVITY OF TRISUBSTITUTED PHENOXYACETIC ACIDS IN THE PEA TEST.

Compound	Concentration in mg./l. to reduce outward curvature by 100°	Relative activity
Indoleacetic acid	0.15	100
2,4,6-Trichlorophenoxyacetic acid	40.0	0.37
2,4,6-Tribromophenoxyacetic acid	> 50.0	*ca.* 0.1
2,4,6-Trimethylphenoxyacetic acid	> 100.0	0.0
2-Methoxy-4,6-dichlorophenoxyacetic acid	*ca.* 40.0	0.4+

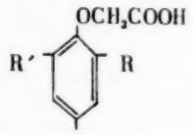
unbuffered. In a few instances the sections were blotted and photographed, but generally the curvatures were simply measured (in laboratory diffuse light) after about 26 hours. The stem reference method (fig. 2 B in **14**) was used for measurement; sections in water give a curvature by this method of about – 150°, and reduction of this negative (*i.e.*, outward) curvature is a delicate test for auxin acivity. The angle was read to the nearest 10°, using a simple protractor. Activities are recorded as percentage of that of indoleacetic acid, solutions of which were always included in the test for calibration. All values quoted are the mean of 14 or 16 curvatures at each concentration, and relative activities are derived from several concentrations.

Results

Activity of ortho substituted compounds

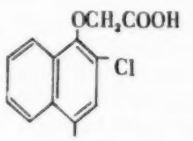
In preliminary tests, the 2,4,6-trichloro-, tribromo- and trimethyl-derivatives of phenoxyacetic acid (synthesized by Dr. Corwin Hansch of Pomona College) were found virtually inactive in the pea test. However, a closer examination of these derivatives and others reveals that they are not en-

tirely inactive. Although these compounds do not produce true inward curvatures, the trichloro-compound (formula I, R = R′ = Cl) at 50 mg./l. reduced the outward curvatures from – 143° (controls in water) to – 33° and the tribromo-compound (formula I, R = R′ = Br) to – 111°. For determining relative activities, the concentration required to reduce the outward curvature by 100° can be used (**15**). Some results are summarized in table I.



R′

I



Cl

II

The trichloro-derivative at least has slight activity, while the activity of the other two compounds is very low or zero. However, clearer activity is shown by 2-methoxy-4,6-dichlorophenoxyacetic acid (kindly supplied by Dr. W. A. Sexton of Imperial Chemical Industries, Manchester, England).

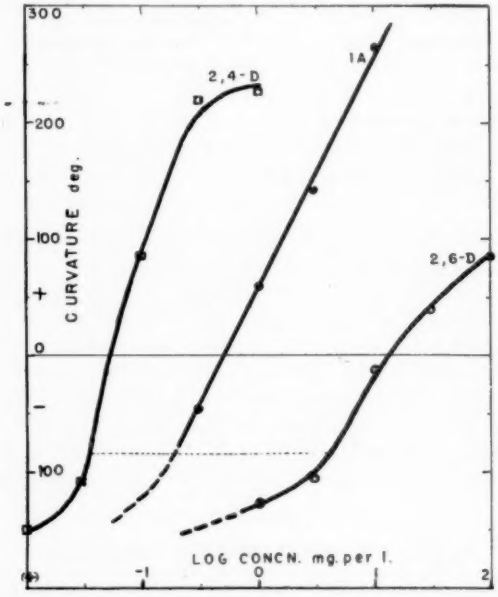
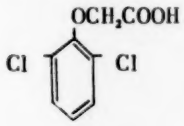


FIG. 1. Curvatures due to 2,6-dichlorophenoxyacetic acid (recrystallized twice), 2,4-dichlorophenoxyacetic acid and indoleacetic acid in the pea test. Each point is the mean of 14 curvatures; all points were determined on the same day. The broken line connects the three concentrations which reduce the outward curvature (– 183°) by 100°.

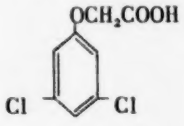
This compound (formula I, R = OCH_3, R′ = Cl) actually causes inward curvature, though only at high concentrations. At 100 mg./l. an inward curvature of 56° corresponded approximately to that caused by indoleacetic acid (IA) at 0.5 mg./l., *i.e.*, a relative activity of 0.5. The figure in table I is based on the reduction of outward curvature by 100°. In other tests the values were 0.35 and 0.65.

Some years ago we had reasoned that it should be possible to increase the low activity of α-naphthoxyacetic acid by halogen substitution. Accordingly, its 2-4-dichloro-derivative, formula II, was synthesized by Dr. G. S. Christiansen in this laboratory and tested. It was, however, almost inactive. At 25 mg./l. the outward curvature was reduced by 140°, and at 10 mg./l. there was no effect. The activity relative to IA is thus a little below 1%. Although the activity of unsubstituted α-naphthoxyacetic acid is very low, it is not zero. It appears, therefore, that the 2,4-chlorination has not appreciably raised the activity. This result, which seemed surprising at the time, supports the view that a position ortho to the side chain must be free.

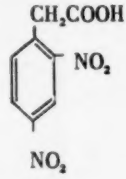
However, the evidence from disubstituted phenoxyacetic acids points in the opposite direction. A sample of 2,6-dichlorophenoxyacetic acid (2,6-D, formula III), kindly supplied by Dr. A. G. Norman, Camp Detrick, gave large inward curvatures (+191° at 30 mg./l. and relative activity of about 4).



III



IV



V

Contamination with a trace of 2,4-D was suspected, especially as SEELEY and WAIN (**11**) reported such contamination in their sample. Accordingly the acid was twice recrystallized from hot benzene. The product (white needles) melted sharply at 133–133.5° C (uncorr.). The melting point reported by SEELEY and WAIN (**11**) is 135–136° C, and LEAPER and BISHOP (**8**) report 137–138° C. The recrystallized product gave the results shown in figure 1 where calibrating curvatures for both indoleacetic acid and 2,4-D, measured on sections from the same lot of plants, are included. By taking the ratios of the concentrations needed to reduce outward curvature by 100°, the 2,6-D has a relative activity of 4.1 (IA = 100); similar ratios for a curvature of 0° give a relative activity of 3.3. These figures are not significantly lower than those of the original sample.

Two other 2,6-D preparations, kindly supplied by Dr. J. M. F. Leaper, Ambler, Pennsylvania, and by Dr. R. L. Wain, Wye, England, have been tested in order to establish this important point. Both were prepared by methods which are stated to exclude contamination with 2,4-D. The prepa-

ration from Wain gave a curvature of + 126° at 100 mg./l. and + 42° at 30 mg./l.; its relative activity (computed from the concentration reducing outward curvature by 100°) is 1.8. The preparation from Leaper gave + 67° at 100 mg./l. and + 8° at 30 mg./l.; the relative activity is 2.2. The compound also promoted the straight growth of pea stems at 10 mg./l. and above, though its relative activity was well below 1% of IA.

While there can thus be no doubt that 2,6-dichlorophenoxyacetic acid is active, 3,5-dichlorophenoxyacetic acid (3,5-D, formula IV), kindly supplied by Dr. A. G. Norman, Camp Detrick, is virtually inactive. Even at 100

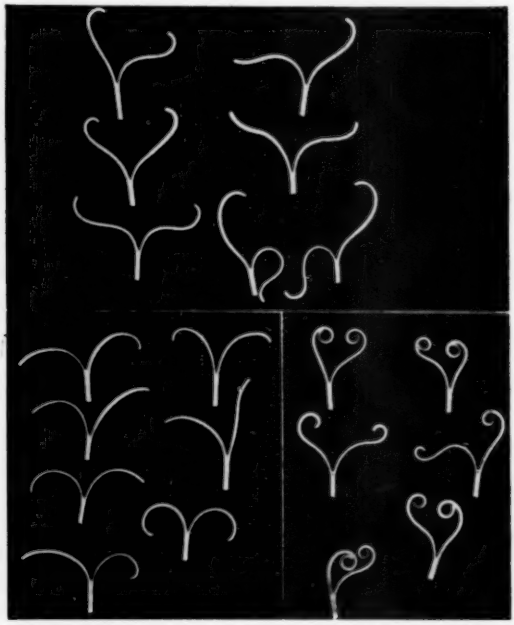


FIG. 2. Curvatures of internodes of pea stems. Top: 2,6-dichlorophenoxyacetic acid at 10 mg./l. (Camp Detrick sample). Bottom left: 3,5-dichlorophenoxyacetic acid at 10 mg./l. Bottom right: 2,4-dichlorophenoxyacetic acid at 10 mg./l.

mg./l. the outward curvature was reduced only 40°. The relative activity is, therefore, very much less than 0.1. Both ortho positions are free in this compound. Figure 2 shows the curvatures caused by pure 2,6-D, 3,5-D, and 2,4-D, all at 10 mg./l. For the sake of completeness a pure sample of 2,5-dichlorophenoxyacetic acid, kindly supplied by Dr. J. M. F. Leaper, Ambler, Pennsylvania, was tested. Its relative activity is about 15.

In striking contrast to the effect of ortho and para halogen substitution is that of nitro substitution. It was found that 2,4-dinitrophenylacetic acid, formula V, was inactive. Even at 100 mg./l. the outward curvature was reduced less than 100°. The relative activity is therefore < 0.1. This

activity compared with the relative activity of 15 for 2,4-dichlorophenylacetic acid (**13**), shows that nitro groups act in the opposite way to halogen atoms in this case.

Activity of benzoic derivatives

Because 2,3,6-trichlorobenzaldehyde had been reported active in the straight growth of Avena coleoptiles (**1**), this compound and the corresponding acid, formula VI, were tested. The compounds were obtained through the kindness of Dr. W. A. Sexton of Imperial Chemical Industries, Manchester, England. The acid is highly active. Its relative activity in

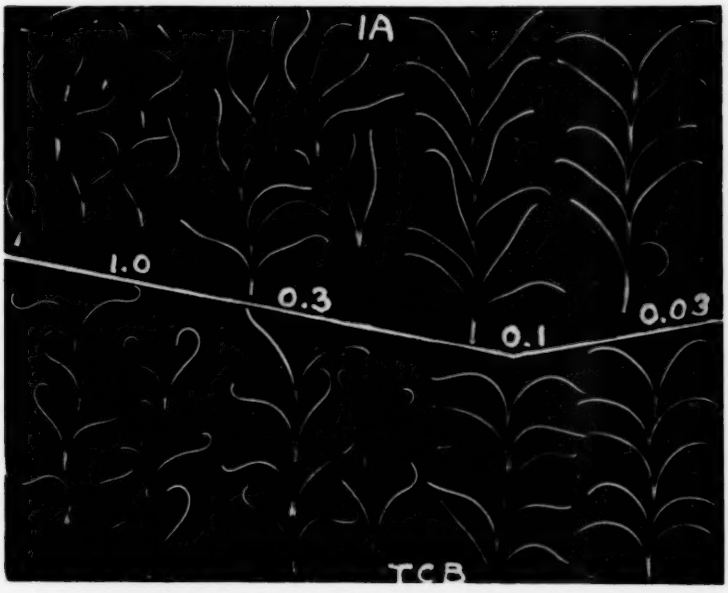
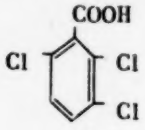


Fig. 3. Comparison of curvatures of internodes of pea stems in indoleacetic acid (IA, above) and 2,3,6-trichlorobenzoic acid (TCB, below). The concentrations (decreasing from left to right) are in mg./l.

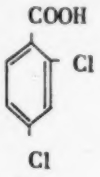
different tests varied from 67 to 360 and averaged about 200; *i.e.*, it is about twice as active as indoleacetic acid. It compares closely with *p*-chlorophenoxyacetic acid, whose relative activity is 200 (**13**). In some experiments, the curvatures were more limited to the apex of the sections than are those due to other auxins. Figure 3 shows a comparison with indoleacetic acid over the critical concentration range. Minute and irregular but real curvatures were produced with the standard agar-block Avena test, at 50 and 100 mg./l.

The activity of the aldehyde is more problematical. The sample as received produced small curvatures which showed no systematic variation

with concentration. Since these might have been due to contamination, it was freed from acidic substances by solution in ether and repeated washing with 0.1 *N* NaOH. However, the material so purified still gave definite curvatures (usually averaging about 0° but in one case as high as +90°) at concentrations of 15 to 200 mg./l.; however, they were not proportional to concentration. As with the acid, the curvatures were markedly apical. Tentatively they must be ascribed to a small degree of conversion of the aldehyde to the acid within the plant tissue itself. Analogous conversion of indoleacetaldehyde has been shown to take place in coleoptile tissue (**7**) and the same reaction has been invoked to explain the activity of naphthaleneacetaldehyde in the pea test (**15**). In a paper which appeared after this work was completed, JONES *et al.* (**4**) report that the aldehyde had greater activity than the acid for inhibiting root elongation. The difference is ascribed to greater permeability of root cells to the aldehyde.



VI



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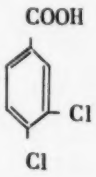

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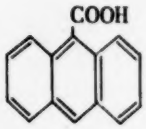

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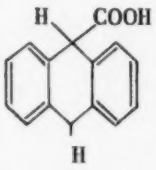

X

The high activity of 2,3,6-trichlorobenzoic acid contrasts with the inactivity of other benzoic derivatives. The related 2,4- and 3,4-dichlorobenzoic acids, formulas VII and VIII respectively, were tested and found inactive. Also 2-chloro-3-nitrobenzoic acid, 2-bromo-3-nitrobenzoic acid and 2-bromo-3,5-dichlorobenzoic acid have been reported inactive in the pea test (**18**). On the other hand, 2,5-dichlorobenzoic acid and 2,6-dichlorobenzoic acid are apparently active on Avena sections (**10**). Lastly, it is worth noting that 2,4,6-trinitrobenzoic acid was found completely inactive in the pea test.

Since in 2,3,6-trichlorobenzoic acid both positions ortho to the carboxyl are substituted, it seemed possible that the requirement for activity of a benzoic derivative might be the opposite of that postulated for phenoxy-compounds. To avoid the complication due to halogen substitution, tests were made of 9-anthroic acid, formula IX, and its 9-10-dihydro-derivative, formula X, synthesized by Mr. Bruce Stowe in these laboratories. Both are

inactive. In the presence of indoleacetic acid they increased the curvature by 50 to 100° (synergistic effect). Although neither was nearly so potent in this respect as triiodobenzoic acid (TIBA) whose synergism was previously reported (**12**), still the synergism is of interest and is greater than that of di-*n*-amylacetic acid (**17**).

Quantitative comparison of synergistic power can be made by determining the concentration of synergist needed to increase the curvature by 100°, using a low (and constant) concentration of indoleacetic acid. Thus with indoleacetic acid at 0.3 mg./l. giving a curvature of −1°, a curvature of +99° was given (by interpolation on a concentration curve) by dihydroanthroic acid at about 3 mg./l., by anthroic acid at above 10 mg./l. and by TIBA at 0.5 mg./l. A more complete comparison is given in table II. The concentrations needed to increase the curvature by 100° for triiodobenzoic acid, 2,6-D and α-phenylbutyric acid were 0.15, 1, and 2–4 mg./l., respectively; for dihydroanthroic acid, anthroic acid and di-*n*-amylacetic acid,

TABLE II

CURVATURES OF PEA STEM SECTIONS IN SOLUTIONS CONTAINING INDOLEACETIC ACID (0.02 mg./l.) AND OTHER COMPOUNDS AT pH 5.5

Compound	Concentration in mg./l. (p.p.m.)							
	0	0.15	0.3	1	3	10	20	30
Triiodobenzoic acid	−67	+77	+99	+98				
9-Anthroic acid	−67				0	+36		+46
9,10-Dihydroanthroic acid	−67			−39	+45			
Di-*n*-amylacetic acid	−67						−21	
γ-Phenyl-butyric acid	−67			−31	+81	+249*		

*Slight auxin activity shown at this concentration without IA.

they were similarly 3, 10, and > 20 mg./l., respectively. These figures can be considered as relative synergistic activities. It should be added that 2,3,6-trichlorobenzaldehyde has no synergistic activity; in the presence of indoleacetic acid, it produces a very slight interference in the curvature response.

Discussion

With the phenoxy acids, the data do not give a very clear-cut distinction between (a) the necessity of a free position ortho to the carboxyl (**2, 3, 9, 10**) and (b) the necessity of two free positions para to one another (**8**). The activity of the 2,6-acid and the real though small activity of the 2,4,6-substituted acids, contrasted with the inactivity of the 3,5-compound, however, favors the latter view. Turning to the benzoic acids, there is support for the suggestion that with this structure the rule might be the opposite of that for the phenoxy acids, namely that two positions para to one another must not be free. The highly active compound 2,3,6-trichlorobenzoic acid has no such positions while the inactive compounds, VII, VIII, IX and X,

each have such a pair (though in IX and X it is in the adjacent nucleus). The compounds 9-anthroic acid and its 9,10-dihydro-derivative have some synergistic activity, but it is noteworthy that TIBA, one of the most powerful of synergists in the pea test (**12**), has no opposite pair. The same is true for 2-bromo-3-nitrobenzoic acid, reported effective as a synergist (**18**). In this connection, it is probable that there is no clear-cut distinction between auxin activity and synergistic activity, since weak auxins like 2,6-D and phenylbutyric acid, as well as many compounds with no true auxin activity, have synergistic effects.

The data of Muir and Hansch (**10, 3**) also offer only quite slender support for the ortho-substitution theory. Thus if electron-attracting groups in the ortho position are necessary to allow of reaction between the benzoic derivative and the postulated plant substrate, then we can see why the 2,6-dichlorobenzoic acid is active but we should expect the 2,4 acid to be active too; it is inactive. If both ortho positions must have electron-attracting groups, then we should not expect the 2,5-dichloro-acid to be active, but it is, and indeed appears to be about 10 times as active (on Avena) as the 2,6 acid (**10**). Also the evidence that chloride is eliminated during growth under the action of 2,6-dichlorobenzoic acid is considerably weakened by the fact that the 2,4-dichloro-derivative, which is inactive, also yields chloride (though admittedly only about half as much). In general the argument of Muir and Hansch is that in spite of the occupation of both ortho positions, activity is still possible through the elimination of chloride. But the real point is that occupation of both ortho positions (plus the meta position) confers activity. For this reason the ortho-substitution theory is felt to be inadequate, at least in its present form.

Other evidence also is unfavorable to this view. In the phenoxyacetic series, substitution of one of the two ortho positions, which might be expected to reduce activity somewhat, greatly increases it, for ortho-chlorophenoxyacetic acid is far more active than phenoxyacetic acid (**13**). In contrast, substitution of the 3 and 5 positions, leaving both ortho positions free, confers no activity at all (fig. 2). As to the inactivation of indoleacetic acid by substitution in the 2 and 4 positions (**2**), this fact, though very interesting, is not critical because all substitutions so far studied greatly reduce the activity of IA. Indoleacetic acid is inactivated for the Avena test by methyl substitution in the 2 and 5 positions (**6**). Mention should also be made of the finding (**6**), again for the Avena test, that the 2-methyl derivative of indoleacetic acid, though much less active than IA, is still four times as active as the N-methyl derivative (in which the ortho positions are free).

If we cannot accept either of these theories we can still consider something which is implied by both of them. The data do indicate that the benzene ring must react chemically with some substrate. The more physical view that the unsaturated ring merely reacts by adsorption to a surface (**16**) does not allow of enough specificity. This fact was already pointed

out (**13**) for the cyclohexene acids, but the present data and other results from aromatic compounds, viewed in a broad way, are much clearer evidence for a direct reaction with the ring carbons. The acetic acid or oxyacetic acid side chain, being electronegative, activates the aromatic ring to react with substituting reagents (especially in the 2 and 4 positions). Correspondingly acetic and oxyacetic derivatives of many ring structures, both aromatic and semi-aromatic, are active as auxins. The carboxyl, being electropositive, deactivates the ring, making it less reactive at all positions. Correspondingly most carboxylic acids (benzoic, naphthoic, indolecarboxylic) are inactive as auxins.

More specifically, in regard to the influence of position of substitution, the data suggest at least a partial interpretation. In the acetic and oxyacetic derivatives, the positions activated are ortho and para, *i.e.*, the **2**, **4**, and 6 positions. The nucleophilic influence is, we may suppose, divided between these three positions, with the result that no one of them is highly reactive. This assumption and the one that follows derive no support from the regular electronic interpretation of organic chemical reactions. However, it must be pointed out that such interpretations have been developed for the conditions prevailing in hot, strongly acid solutions. Modification of them for the cold, nearly neutral conditions in the plant does not seem unwarranted if it results in a consistent theory. In this case the interpretations fit well with the observation that phenylacetic acid has a relative activity of only about 3, and phenoxyacetic acid of less than 1. Occupation of one or more of these positions, it is suggested, leads to intensification of the nucleophilic (or activating) influence at the remainder. Correspondingly substitution at either the 2 or the 4 position by Cl or CH_3 increases auxin activity, and this effect is greatest with disubstitution at **2** and **4**, which concentrates all the ability to react into the remaining 6 position. However, if all three activated positions are occupied, no possibility of reaction remains; the compounds have a very small activity which is no more than that of unsubstituted phenoxyacetic acid. In this connection the relative activity of 5% formerly ascribed to pentachlorophenoxyacetic acid (**13**) is now found to be due to an impurity. The relative activity of a carefully purified sample is less than 0.1%. Partial hydrolysis may make some activity possible. When the 3 and 5 positions, which are not activated, are occupied, the distribution of activation between the 2, 4, and 6 positions is not affected. This compound is no more active than the unsubstituted parent compound. Since 4-chloro-phenoxyacetic acid is much more active than the 2-chloro-acid, it seems that the ortho position is more strongly activated than the para. This observation can then explain the low activity of the 2,6-dichloro-compound in which both of the two most active positions are occupied.

In the benzoic acids, it was shown that they are made active as auxins only by substitution with more than one halogen. Now these halogens by their resonance allow of activity in the positions ortho and para to them-

selves, not to the carboxyl. Hence in 2,6-dichlorobenzoic acid the 3 and 5 positions are the ones which remain active and may give some auxin activity; but if we now substitute the 3 position as well, the activating influence is concentrated in the 5 position and gives highest activity. It can be predicted that the 2,3,5,6-tetra-substituted acid should be of low activity.

It is necessary to make the reservation that the combining positions must not react too strongly. Hydroxyl groups, which strongly activate the ring, invariably reduce auxin activity (so far as known). Methyl groups, which activate less strongly, increase it. On the other hand, halogen atoms, which clearly confer auxin activity, are known actually to deactivate the ring somewhat; and the auxin activity of chloride compounds must be due largely to their resonance which has the effect of concentrating the reactivity of the ring at certain points. Strong deactivation, as with the positive nitro group, does not confer activity, and may (as in 2,4-dinitrophenylacetic acid) remove the activity of the parent substance. The two most effective substituents of auxins, namely CH_3 and Cl, are (from the chemical viewpoint) the weakest agents in activating and deactivating respectively. In other words they are the two that come nearest to the ideal of simply occupying a position, without too greatly affecting the general reactivity of the ring. It is easy to visualize how the loose relationship that has to exist between an enzyme and its activator (coenzyme, protective agent, etc.) would call for a moderate, but rather closely-defined, degree of reactivity.

If the above views are correct the ring does combine (though loosely) with a substrate; but the point of combination is not limited to the ortho position, and in fact varies with the substitution on the ring. The substrate seems most likely to be weakly positively charged. These views can to some extent be tested and it is hoped to do so in the near future.

Summary

Three derivatives of phenoxyacetic acid in which both positions ortho to the side chain are substituted, were found active as auxins in the pea test. These are 2,6-dichloro-, 2,4,6-trichloro- and 2-methoxy-4,6-dichloro-phenoxyacetic acids. The 3,5-dichloro-derivative, however, is quite inactive. In phenylacetic acid, while 2,4-dichloro-substitution increases activity, the corresponding 2,4-dinitro-substitution decreases it.

The compound 2,3,6-trichlorobenzoic acid is highly active, more so than indoleacetic acid, but the activity of the aldehyde is very much lower and does not vary uniformly with concentration. For this reason its slight activity is considered to be due to its conversion to the acid in pea stem tissue. Two other chlorinated benzoic acids were found inactive.

The activities of several other compounds are reported. Several compounds, including some with and some without auxin activity, are shown to have synergistic action in the pea test.

The results are discussed in the light of two proposed new rules for the relation between structure and activity. It is concluded that neither of

these rules, namely, (a) that activity requires two free positions para to one another, and (b) that activity requires one free position ortho to the carboxyl, is consonant with all the facts, though some evidence, both here and in the literature, favors each of them. There is also some indication that benzoic acid derivatives may obey an opposite rule to that for phenoxyacetic and phenylacetic acid derivatives.

Arguments in favor of the conclusion that a definite chemical reaction does take place at one or more positions on the ring are summarized. It is considered that this general conclusion is consonant with all the data. A detailed theory, to account for the powerful effects of specific substitutions, is proposed.

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The author's thanks are due to Mrs. Irma Slankis for assistance with the tests. He is also grateful to Prof. Louis Fieser and Dr. Arvid Ek for critical discussions.

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BRIEF PAPERS

FURTHER STUDIES IN THE SIMPLIFICATION OF EQUIPMENT FOR SUBIRRIGATION EXPERIMENTS IN PLANT NUTRITION

GEORGE R. JOHNSTONE

(WITH TWO FIGURES)

Received August 2, 1951

A new type of equipment for use in subirrigation experiments in plant nutrition has the special advantage of being enlarged to accommodate larger plants without decreasing the convenience of operation. A brief description of the older equipment will precede that of the newer equipment for the convenience of comparison.

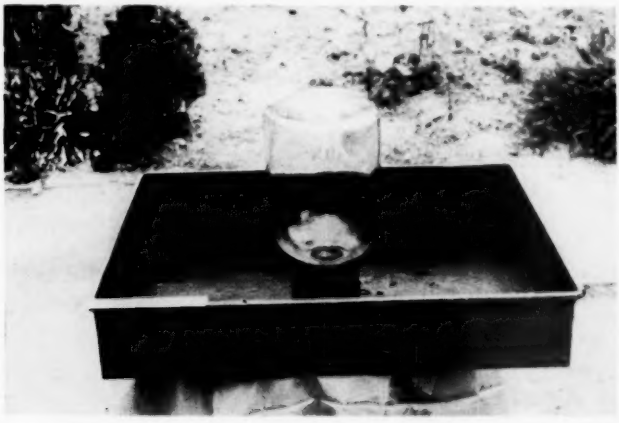

FIG. 1. Sand culture equipment. The gallon bottle reservoir was subsequently coated with aluminum paint. The magnification is indicated by a six inch ruler, left.

The previously described equipment for subirrigation experiments in plant nutrition (**2**) has certain advantages as well as limitations. This equipment comprises two compartments, superimposed one above the other. The lower compartment serves as a reservoir for the nutrient solution, while the upper compartment filled with highly insoluble material provides the seed bed. The lower compartment is equipped with a centrally placed plastic tube filled with the same kind of medium as that in the upper compartment. This plastic tube, one and one fourth inches in diameter, provided with a plastic cap over the lower end, and with lateral perforations,

serves as a pathway for the migration of the nutrient solution to the seed bed. Small containers having a diameter of six inches are convenient to operate, but limited to the culture of small plants, such as *Impatiens Balsamina, Saintpaulia ionantha,* and *Capsicum frutescens* var. *longum.*

The new type of equipment can be increased in size to accommodate larger plants, such as the tomato, *Lycopersicum esculentum,* without decreasing particularly its convenience of operation. The plan of this apparatus is essentially different from the above described equipment in its reversal of the positional relation of the reservoir to the seed bed. In this

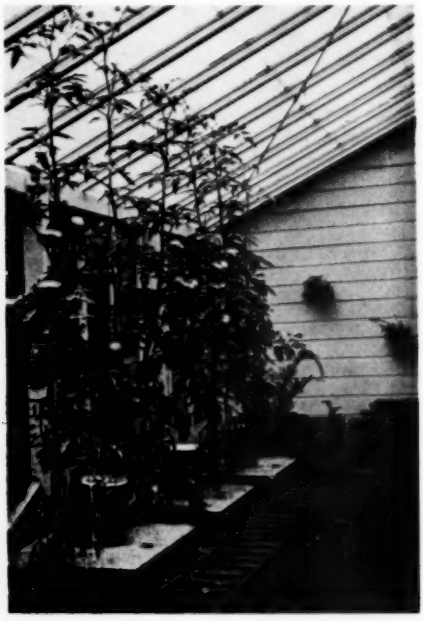

FIG. 2. Sand cultures of San Jose Canner tomato plants: left, K high, K_2SO_4 substituted for $(NH_4)_2SO_4$; center, control, a Rubidoix solution (1); right, KCl substituted for $(NH_4)_2SO_4$. Age 93 days. A cylinder with a stopper in front of each reservoir is used when siphoning off the solution or when flushing with water.

new equipment the reservoir is above the seed bed. An inverted one-gallon bottle coated with aluminum paint to exclude light is used as the reservoir. This bottle is held in position by an asphalt-coated tin cylinder. The length of the cylinder is adjusted so that the mouth of the inverted bottle is one half inch or less from the bottom of the seed bed. Such an apparatus (fig. 1) has been used successfully for the culture of San Jose Canner tomato plants (fig. 2).

The operation of this apparatus is relatively simple. Four inches of clean, 12-mesh, silica sand comprise the seed bed. The sand is moistened

by capillary rise of water from the subirrigated layer of half strength nutrient solution including the minor elements. The half strength nutrient solution is preferred during the first three weeks for the culture of San Jose Canner tomatoes. As evaporation tends to concentrate the nutrient solution, the water lost by transpiration and direct evaporation from the sand is replaced by adding distilled water, or tap water if less accurate work is satisfactory. The city water department may furnish the analysis of the tap water which may justify some modification of the amounts of minor elements employed.

After three weeks in the half strength nutrient solution, the solution may be siphoned out and the seed bed flushed with distilled water, and replaced with a full strength nutrient solution. Best results may be obtained by completely changing the nutrient solution after flushing out the old solution every six weeks or oftener if the plants are large.

Some of the disadvantages of this equipment, such as variations in the concentration of the nutrient solution because of evaporation and replacement of the water thus lost, unknown quantities of chemicals which adhere to the sand particles, and desired flushing of the seed bed and complete change of the nutrient solution are not considered serious because many features of this system resemble those which occur under natural conditions. Some of the advantages of the system are obvious, such as the convenience of operation and the apparent reduced cost of the equipment.

Acknowledgment is hereby accorded Harris C. Moore for his help in the experimental work, the results of which shall appear elsewhere.

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RELATIONSHIP OF THE COBALT AND LIGHT EFFECTS ON EXPANSION OF ETIOLATED BEAN LEAF DISKS[1]

CARLOS O. MILLER[2]

(WITH THREE FIGURES)

Received September 20, 1951

The expansion of etiolated bean leaf disks in the dark may be promoted by briefly exposing the disks to light. Expansion may also be increased by placing the disks on media containing cobaltous or nickelous salts (**1**) and the author has suggested a close relationship of the light and cobalt effects. Investigations concerning this relationship are reported herein.

A full description of the test utilizing the etiolated leaf disks has been published (**1**) and is recorded elsewhere (**2**). It is summarized here. Disks, 5.0 mm. in diameter, are cut with a cork borer from the two simple leaves immediately above the cotyledons of etiolated Burpee Dwarf Stringless Greenpod bean plants. A section of a main lateral vein approximates a diameter in each disk. The plants are used seven to nine days after the sowing of the seeds in flats of sand in a darkroom maintained at a temperature of $25 \pm 1°$ C. All manipulations involving the disks are performed in dim green light. Five milliliters of a test solution are added to a Petri dish containing three 9 cm. sheets of Whatman no. 1 filter paper. Ten disks, lower epidermis up, are placed on the pad of filter paper wetted with the test solution. The basal solution contains 3% D-glucose by weight and KNO_3 at an 0.08 M concentration. The pH of each solution is adjusted to 5.6. The Petri dishes are kept in a dark cabinet at $25 \pm 1°$ C. The diameters, perpendicular to the segments of heavy veins, are remeasured at the desired times.

In figure 1, the values for diameters of disks two days after light exposures are plotted against the length of time the disks were exposed to incandescent-filament light at an intensity of 200 foot-candles. The greatest increase per amount of light energy occurs with exposures of five minutes or less. Actually, as found in other experiments, the sharp rise of the curve occurs with exposures of one minute or less. The curve does not level off at a maximum value. It probably would continue upward with longer exposures since expansion of disks exposed to light at the beginning of several experiments was considerably further increased by another exposure the next day. Thus, the limiting condition affected by light is not completely eliminated by a single exposure to light. After all light treatments, expansion was nearly uniform in all directions.

[1] Papers from the Department of Botany and Plant Pathology, The Ohio State University, no. 535.

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As previously reported (**1**), the expansion of disks on a cobalt-containing medium was nearly uniform along all diameters. The optimal concentration was about 5 p.p.m. of the cobaltous ion.

The mechanics of the disk expansion after both light and/or cobalt treatments was investigated. Cell size measurements were made in sections prepared from material mounted in paraffin. Practically all expansion was apparently a result of cell enlargement. Therefore, both cobalt and light influence processes which lead to cell enlargement.

If the cobalt and light actions are distinctly different, it should be possible to find plant material in which one effect could be observed and the other could not be. In search of such material, disks from leaves of plants growing in the greenhouse were employed as test objects. The basal medium already described was used. The expansion of disks from green primary bean leaves having areas of about 8 sq. cm. increased in the presence of cobaltous ions; however, the expansion also increased after the disks were exposed to light (200 fc) for five minutes one day following the start of the tests. The same appeared to be true of leaf disks from young green pea

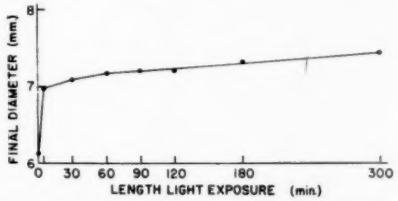

FIG. 1. Final diameters of etiolated bean leaf disks after two days in darkness on basal medium. At the beginning of the experiment disks exposed to light (200 fc) for the time indicated. Standard errors ranged from ± 0.03 to ± 0.07 mm.

plants (var. Little Marvel) although the results were not very clear-cut since not much growth occurred. The expansion of disks from green leaves of *Chenopodium album* and of radish (var. French Breakfast) was unaffected by cobalt and by exposure to light. This was true of *Chenopodium album* leaf disks even if coumarin was present in the medium (**3**). Thus, no separation of the two effects was obtained by use of green leaf material.

Shortly after germination, a hook is evident in the hypocotyl of an etiolated bean seedling. Under conditions of these experiments, as the seedling grew, this hook moved morphologically upward from the hypocotyl into the first internode of the stem and finally into the petioles of the primary leaves. Petioles of both primary leaves became hooked. Exposure of the seedlings to light caused the disappearance of the hooks. In several experiments, hooked portions of hypocotyls, stems, and petioles were excised from the seedlings and used as test objects. Although exacting techniques were not developed, it was obvious that the elongation and straightening of the hooks could be increased by exposing them briefly to light or by adding cobaltous

salts to the basal medium. The same was true of any excised portions taken from above but not from below the bend. However, these latter portions grew very little; therefore, the tests made to detect light and cobalt effects may not have been valid. This is a further example of association of the cobalt and light effects. In no tissue has only one of the effects been observed.

Distinctly different causes, if existent, of the cobalt and light effects might be reflected in the time-expansion curves. In figure 2, time-expansion curves are presented for disks exposed for 15 min. to incandescent-filament light at an intensity of 200 foot-candles and placed on a basal medium containing 5 p.p.m. Co^{++} (as $Co(NO_3)_2 \cdot 6\,H_2O$), for disks not exposed to light

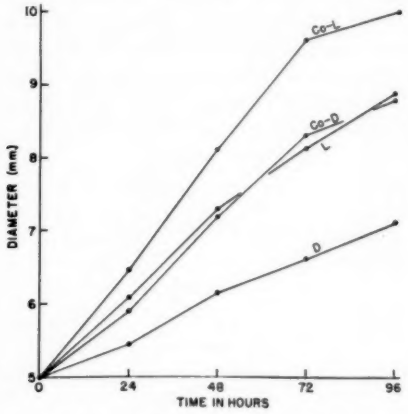

FIG. 2. Time course of expansion of etiolated bean leaf disks given the following treatments: D—no light exposure, basal medium; CoD—no light exposure, Co^{++} (5 p.p.m.) in basal medium; L—15 min. light exposure, basal medium; CoL—15 min. light exposure, Co^{++} in basal medium. Standard errors ranged from $\pm$ 0.03 to $\pm$ 0.07 mm.

but placed on the cobalt salt medium, for disks exposed to light and placed on basal medium, and for unexposed disks placed on basal medium. Except for the steepness of the slopes, there are no characteristic differences among the four curves; they are in phase. Quantitatively, the cobalt-dark (Co-D) and light (L) curves were similar in other experiments but not so identical as those presented in figure 2. The curves in figure 2 bring out no distinction between the cobalt and light effects.

The disks given both cobalt and light treatments, however, expanded more than disks given either treatment alone even though the optimal concentration of cobaltous salt was used. Obviously, the action of Co^{++} does not replace the action of light. From the curves it seems the two effects might be additive. This possibility was tested.

A wide range of values for expansion of disks given one or both treat-

ments was obtained by measuring diameters of disks at various times after the start of experiments, by varying the length of light exposure, and by varying the concentration of cobaltous salts in solution. The amount of expansion resulting from any particular treatment was obtained by subtracting the computed areas of the control disks (given neither cobalt nor light treatment) from the computed areas of the treated disks. In figure 3, values along the ordinate represent the increases in area of disks given both cobalt and light treatments. The values along the abscissa represent the sums of area increases resulting from the cobalt and light treatments being given separately to different disks. If the effects are strictly additive, the points should fall on the theoretical line which has been drawn in the figure.

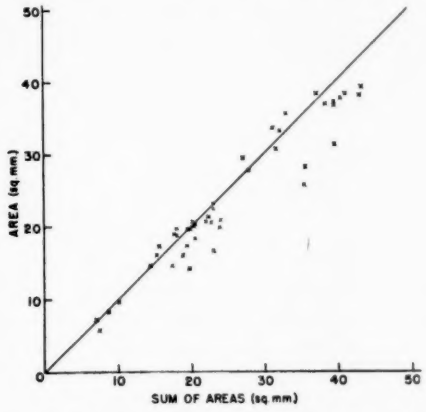

FIG. 3. Increases in etiolated bean leaf disk areas resulting from Co^{++} and light treatments given simultaneously to same disks vs. the sums of area increases resulting from treatments given separately to different disks. See text for explanation of the curve.

The deviations are not great. The cobalt and light effects, at least under the conditions of the experiments reported here, are essentially additive.

The cobalt and light effects are similar in that both occur in the same plant materials, both increase cell enlargement, both cause uniform leaf disk expansion, and both influence some property apparently common to cells in many different tissues. These relationships support the idea that both cobalt and light affect the same limiting condition. It might be argued that the fact that the two effects are additive is evidence against such a possibility. This argument would not necessarily be valid. The limiting condition affected by light was only partially removed by single exposures of the disks to light, and addition of cobaltous ions might further diminish the same condition. It seems quite possible that cobalt and light reduce the same limiting condition but perhaps by different pathways.

The author wishes to thank Dr. B. S. Meyer for his helpful guidance during the course of this work.

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LIGHT ABSORPTION BY CHLOROPHYLL AT HIGH CONCENTRATIONS[1]

S. Aronoff

(WITH TWO FIGURES)

Received September 27, 1951

It is well known that the absorption spectrum of the chlorophylls in a leaf is displaced to the red when compared to that in a non-biological system. Apart from purely physical considerations, three types of chemical reactions may result in such a shift: (a) the reaction of chlorophyll and solvent (refractive index effect); (b) the reaction of chlorophyll and some other solute (adsorption or compound formation); (c) the reaction of chlorophyll molecules among themselves (polymerization). In this paper evidence is presented which indicates that the spectral shift is probably not due to polymerization of the chlorophyll molecules. Polymerization may result in bathychromic shifts, and previous studies of chlorophyll spectra have dealt with relatively dilute solutions (10^{-3} M or less) while the concentration of chlorophyll in the leaf is of the order of 10^{-1} M. In the absence of density values for the chlorophylls one may assume, for purposes of discussion, that the pure solid chlorophylls are approximately 10^{0} M.

All spectra were obtained using a Beckman spectrophotometer. The crude chlorophyll was prepared from dried spinach, precipitated from petroleum ether, and dried in high vacuum. The presence of carotenoids was felt to be immaterial in view of the region of the spectrum utilized. Purified chlorophyll a was prepared by chromatography of the crude chlorophylls on sucrose columns (**5**).

Dilutions of the crude chlorophyll were made by dissolving the weighed amount of chlorophyll in a minimal amount of diethyl ether and mixing this solution with a known weight of Celvacene light vacuum grease. The ether was removed in vacuum until there was no detectable odor of ether and the grease appeared to have regained its original viscosity. The solution of chlorophyll in the grease was spread on clear glass plate in a uniformly thin layer, of a thickness such that the resulting optical density was 0.2 to 0.6. The Celvacene had negligible absorption in the region employed. Absorption corrections were required for the glass plate, which was three sixteenths of an inch thick, rectangular, and of such dimensions as to fit snugly within the absorption cuvette.

The blank for the living leaf was tobacco leaf tissue from which the chlorophyll had been extracted with 95% ethanol.

[1] Contribution no. 154 from the Institute for Atomic Research and the Department of Botany, Iowa State College, Ames, Iowa. This work was supported by AEC contract no. AT(11-1)-59.

The data of figure 1 show that increasing the concentration of chlorophyll in solutions does not shift the position of the red absorption maximum to coincide with that for the living tobacco leaf. The position of the maximum in the leaf is not of itself sufficient evidence for an association of the chlorophyll in the leaf since there is a variation of the position according to the solvent employed (**1, 2**). Nevertheless, there is no solvent in which chlorophyll has the same region of maximum absorption as it has in the leaf. The possibility that the solvent matrix within the leaf is of a character

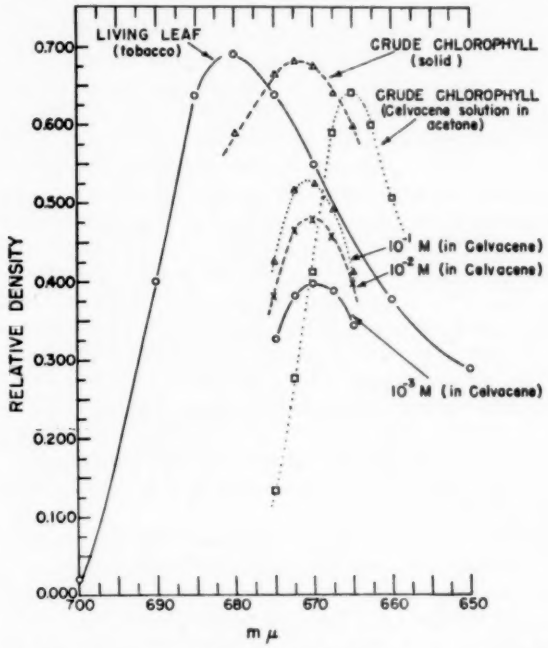

FIG. 1. Absorption maxima of crude chlorophyll at various concentrations and in different media. Curves are transposed for clarity. In the leaf the effective band width was 35 Å, whereas in the rest of the data it was 12 Å.

(presumably refractive index) far different from any solvent which has been used is rather improbable in view of the range of materials used and the data presented here. The possibility that the chlorophyll is associated with some substance in the leaf is more probable in view of the ease of preparation of chlorophyll-protein complexes from leaves and microörganisms. Furthermore, synthetic chlorophyll-protein complexes may also be made (**3**) which exhibit most of the solubility characteristics associated with the biological system. Nevertheless, it is known that metalloporphyrins may combine with proteins and, until proved otherwise, the chlorophyll-protein may

have resulted merely from proximity during the mechanical manipulation. The question of the chlorophyll/protein ratio in chloroplasts has been discussed by RABINOWITCH (4). Considerations of the spatial requirements of the various substances within a chloroplast indicate there may be no room for protein of the dimensions usually associated with that substance. If the protein is within the grana, it may be considerably less folded than normal; if, however, it exists within the stroma of the plastid, then on extraction a chlorophyll-protein adsorption may result. The primary interest in such a protein arises from its possible utilization in hydrogen transfer during photoreductive processes.

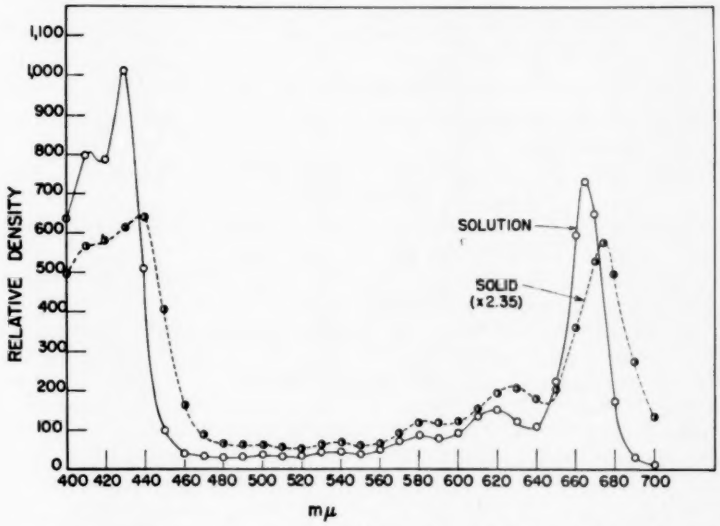

FIG. 2. Absorption spectra of solid chlorophyll a and the equivalent solution in acetone. The absorption coefficients for the solid have been multiplied by 2.35 to transpose the solid curve to a height comparable with that of the solution.

The most positive evidence for compound formation between chlorophyll and protein is the enzymatic activity of the complex such as that shown in the Hill reaction. The water-soluble sodium, magnesium-chlorophyllin is, by itself, inactive with a variety of substrates (unpublished observations). However, no attempts to prepare a synthetic chlorophyll-protein complex which would perform a Hill reaction appear to have been made.

In the experiments with purified chlorophyll a it was possible to obtain comparable spectra of the solid substance and its solution (fig. 2). The pigment was transferred quantitatively with acetone from the plate to the cuvette, the latter being filled with solvent to the same height as the pigment was on the plate. The light path consequently traversed the same number of mole-

cules in both measurements. Knowledge of the extinction coefficients of the solution permitted calculation of the extinction coefficients of the solid. In general the solid coefficients were approximately one third those of the solution. Since similar diminutions in intensities could be shown with the samples concentrated in grease (for the 10^{-1} M concentration the relative values for the solid and the solution at the red maximum were 62 and 100), a problem is posed as to the assignment of valid absorption coefficients to the pigments within the leaf. These values are of considerable interest since they are used of necessity in calculating the efficiency of utilization of absorbed energy.

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THE EFFECT OF HYPOCOTYL TEMPERATURE ON TRANSLOCATION OF CARBOHYDRATES FROM BEAN LEAVES[1]

R. H. Böhning, C. A. Swanson and A. J. Linck

(WITH TWO FIGURES)

Received October 1, 1951

In a recent paper, Swanson and Böhning (**2**) reported the influence of petiole temperature on the rate of carbohydrate transport from bean leaves, as measured by the rate of stem and leaf elongation in complete darkness. Sugar was supplied to the plants by immersing the blade of the petiole-jacketed leaf in 0.75 M sucrose solution. An optimal petiole temperature for transport of near 25° C was indicated in these experiments. No evidence was found of a negative temperature coefficient (a Q_{10} less than 1) such as Went and Hull (**3**) reported for tomato in the range from 5 to 30° C.

These studies have now been extended to include the influence of hypocotyl temperature on the rate of carbohydrate transport from bean leaves, using a similar technical approach.

Seeds of *Phaseolus vulgaris*, variety Black Valentine, were planted in sand and allowed to germinate under greenhouse conditions. As soon as the hypocotyls had straightened, selected seedlings were transplanted to a culture tray containing a complete culture solution. The plants were individually supported by means of split cork stoppers placed around the base of the hypocotyl. The solution was continuously aerated and the plants were allowed to develop under the prevailing greenhouse conditions until used in the experiments.

When the primary leaves were nearly expanded and the distance from the base of the hypocotyl to the primary leaf node was approximately five inches, the plants were transferred to specially constructed temperature jackets mounted above asphalt-paraffin-coated metal trays containing complete culture solution and equipped with carbon tube aerators. Each of these units accommodated 10 plants, and four such units were used in each experiment. A modification was made in some of the later experiments in that in place of the metal trays, the roots of each plant were allowed to develop in aerated culture solution in individual glass tubes. The jackets for temperature control were larger ($4'' \times 4\frac{1}{4}'' \times 41\frac{1}{2}''$) than those used in controlling petiole temperature (**2**), but afforded the same flexibility and accuracy of control. The temperature of the hypocotyl was varied within the range of 7 to 40° C by means of the jackets while the temperature of the rest of the plant was maintained at 25 ± 1° C.

[1] Papers from the Department of Botany and Plant Pathology, The Ohio State University, Columbus 10, Ohio. Number 537.

Initial measurements of the length of the stem (from primary leaf node to tip of the youngest bud) were made as soon as the hypocotyls had been jacketed. In certain experiments the plant units were set up in a controlled temperature room equipped with a bank of 12 slimline fluorescent lights

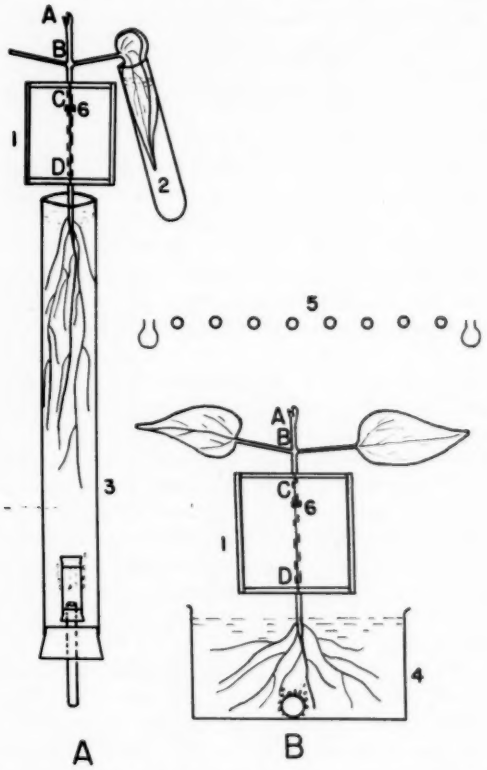

FIG. 1. Diagram of experimental set-up. Method A: rate of translocation of nutrients from a leaf supplied with sugar measured by rate of elongation of stem AB in complete darkness as a function of the temperature of stem-hypocotyl CD. Method B: rate of translocation of photosynthate from primary leaves measured by rate of elongation of stem AB. 1, temperature jacket; 2, sucrose solution; 3, culture tube; 4, culture tank; 5, adjustable light bank; 6, cotyledonary node.

supplemented with 14 60-watt mazda lamps. This adjustable bank of lights was maintained at a distance of approximately one foot above the growing tips of the plants and supplied a light intensity of about 1000 foot candles.

In other experiments the plants were allowed to develop in the controlled temperature room without artificial illumination, carbohydrate being sup-

plied by immersion of one primary leaf blade in 0.75 M sucrose solution. In these experiments, eight plants in each unit received the sugar solution and two plants in each unit served as checks. Periodical measurements of the growth of the stems were made throughout the course of the experiments.

A diagram of the experimental set-up is shown in figure 1. Although the major region exposed to the various temperature regimes was the hypocotyl, it will be noted from the diagram that a small segment of the stem was also enclosed in the temperature jackets. For the sake of brevity, the term

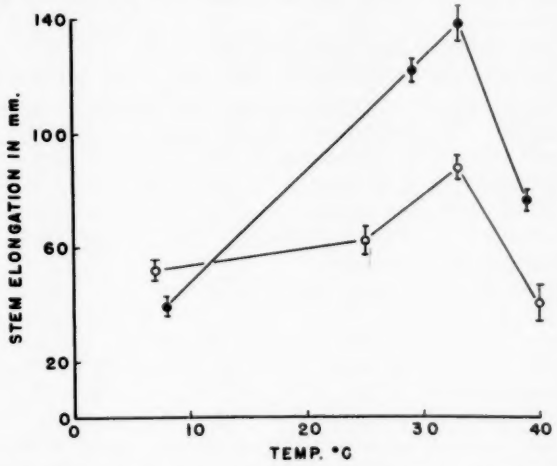

FIG. 2. Results of two typical experiments showing the effect of hypocotyl temperature on stem elongation. ○—○ represents growth of stem of sugar-supplied plants less elongation of the check plants. (Duration of experiment 115 hours.) Each point represents the average of eight plants. Elongation of stem of check plants in experiments of this type was very small (average for experiment reported was only 13 mm). ●—● plants artificially illuminated. (Duration of experiment 166 hours.) Each point represents the average of 10 plants. Standard error is indicated by the vertical lines through each point.

hypocotyl is used to refer to the stem-hypocotyl segment CD which was jacketed.

The results of a typical experiment employing each of these methods (fig. 1, A and B) are presented graphically in figure 2. Although the absolute values of stem elongation varied from one experiment to another the data of each experiment when graphed resulted in a curve similar to those presented. As may be noted from these data, hypocotyl temperature markedly affected the elongation of the stem AB (fig. 1), the effect being similar to that of petiole temperature observed in earlier studies (**2**). Maximum transport of both naturally occurring and artificially supplied carbohydrate,

as measured by stem elongation, occurred at a temperature of approximately 33° C.

Since the rate of elongation of the stem may be expected to be limited in part by the rate of supply of food materials from the primary leaf to the stem tip, it may be inferred that hypocotyl temperature affects the rate of this translocation. This remote control of hypocotyl temperature on food export from the leaves is more clearly deduced from the experiments of Method A (fig. 1). In Method B, the effect of temperature is apparently much more marked, but the effect is undoubtedly exaggerated due to the increased photosynthetic area resulting from expansion of the trifoliate leaves. The young shoots at a hypocotyl temperature of 33° C have an advantage during the early phase of growth while they are still primarily dependent for food on the primary leaves, this temperature being optimal for food export. This initial gain is rapidly capitalized on by the exponential increase occurring in the photosynthetic area of the developing shoot. Differences in growth of the stems under the various treatments would thus become greater with time due to the variability in carbohydrate supply brought about by differences in photosynthetic area as well as to the effect of temperature on translocation.

The effect of localized temperature differentials on the rate of transport through parts of the plant remote from the treated portion may also be inferred from the data of DAY (**1**). Day showed that killing a section of the hypocotyl with steam delayed the translocation of 2,4-D from the leaf to the epicotyl and reduced the amount translocated. From these facts and other data he concluded that interruption of the phloem at any point on the stem or petiole interferes with downward transport at all other points on the petiole or stem. The present data enlarge on this concept by suggesting that hypocotyl temperatures, within physiological limits (7 to 40° C) but ranging from limiting to inhibitory levels, affect phloem transport accordingly in all other parts of the plant.

The present technique has not proved adequate in assessing the effect of hypocotyl temperature on the rate of food transport to the roots. No reproducible and statistically significant trend in root growth (maximum elongation, fresh weight, or dry weight) was observed which could be related to hypocotyl temperature. In experiments using Method B, root growth (maximum lineal extension) of the controls was only 20 to 30 mm. less on the average than in the case of the sugar-supplied plants. This range was too small to establish differential effects of temperature.

The inference from the above data that phloem transport along a given path is influenced by temperature differentials applied outside that path is compatible with the view that translocation in the phloem is mainly by mass flow of solutions. Such a mechanism of translocation in the phloem would envisage a unit hydraulic system in which the effect of a given factor applied at any point would be transmitted throughout the entire system. Alternative hypotheses cannot be ruled out, however. It is possible that

elongation of the stems was limited by a compound synthesized in the roots. In this case, however, it would be necessary to further postulate that the synthesis of this compound was in turn limited by the rate of transport of a precursor from the leaf to the roots, for the rate of stem elongation in the minus-sugar controls was nearly negligible.

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THE BIOSYNTHESIS OF CARBON-14-LABELED COMPOUNDS. II. THE CHROMATOGRAPHIC SEPARATION OF THE MONOSACCHARIDES, DISACCHARIDES, AND TRISACCHARIDES FROM PLANT EXTRACTS[1]

G. R. Noggle and M. Eleanor Schumacher

(WITH ONE FIGURE)

Received November 16, 1951

During the development of a method for the separation of biosynthetically prepared C^{14}-labeled glucose and fructose (**4**), it was found necessary to remove sucrose from the plant extracts before the monosaccharide separation could be effected. Montgomery *et al.* (**3**) used the Tiselius displacement technique for isolating 6-(*a*-D-glucopyranosyl)-D-glucose from an enzymatic starch hydrolyzate, and indicated in a footnote that the method could be adapted to a separation of monosaccharides and disaccharides. Whistler and Durso (**7**) demonstrated that a mixture of charcoal (Darco G-60) and Celite (Celite 535) could be used for the column separation of monosaccharides, disaccharides, and trisaccharides. Using the former technique, we found that glucose and sucrose could be separated but that the method involved the use of three charcoal columns in series, and rather large volumes of solution were required to carry out the separation. The latter method does not need elaborate equipment and enables one to handle rather large amounts of sugar without resorting to excessively large volumes of desorbing solutions. It was found possible, by using this method to separate sucrose from the monosaccharide fraction of plant sugar extracts and, in addition, to isolate a trisaccharide fraction.

The general procedure followed is that of Whistler and Durso (**7**) and is here briefly summarized. A mixture of Darco G-60 and Celite 535 was selected as the adsorbent. Other charcoals were tried but the Darco G-60 gave the most consistent results. Equal amounts by weight of the charcoal and Celite were mixed, washed with water, filtered, and dried. The adsorbent (35 gm.) was packed dry in a glass chromatographic tube 25 mm. in diameter and 300 mm. long, the bottom of which was attached to a receiving flask, which in turn was connected to a vacuum line. The adsorbent was slowly added to the tube (vacuum on) until a column 250 mm. long was attained. During the filling process the sides of the tube were vigorously tapped to aid in packing the adsorbent. Before the addition of the sugar solution 150 ml. of water were passed through the column, the flow rate then being maintained at approximately 200 ml. per hour. Whistler

[1] Work performed under contract no. W-7405-eng-26 for the Atomic Energy Commission.

and Durso state that a column 34 mm. × 230 mm. has a loading capacity of 2 gm. of sugar. Our columns had roughly the same capacity but the amount of sugar placed on the column was dependent upon the relative proportions of monosaccharides and disaccharides present in the solution. If the disaccharide fraction predominated, then not more than 1 gm. of sugar was loaded on the column. If the disaccharide fraction was small then up to 2 gm. of sugar could be put on the column.

The sugars were removed from the column by the successive additions of water and increasing concentrations of ethanol. The monosaccharide fraction was desorbed with 800 ml. of water, the disaccharide fraction with one liter of 5% ethanol, and the trisaccharide fraction with 500 ml. of 15% ethanol. From the time that the adsorbent was wetted with water, the sugar solution and desorbing solutions were so added that the column was not permitted to run dry. The columns could not be reused and a new one was prepared for each separation.

The desorbed sugar solutions were concentrated and aliquots analyzed for reducing sugar and total sugar after acid hydrolysis. Samples of the aliquots were also run on paper chromatograms (6) to check on the completeness of separation. Radioactive sugars were detected on the paper chromatograms by exposing them to Eastman No-Screen X-ray film.

We found, as did Whistler and Durso, that the monosaccharides were desorbed with 800 ml. of water. A smaller volume of water decreased the recoveries of the monosaccharides, while a larger volume increased the chance for the disaccharide fraction to appear as a contaminant. Occasionally, however, small amounts of disaccharides were found in the monosaccharide fraction when 800 ml. of water was used. Most of the difficulty was found to be due to inadequately mixed charcoal and Celite. As the mixture had a tendency to layer out when stored, a column containing an excessively high proportion of Celite was occasionally prepared. Under these conditions the disaccharide fraction appeared in the water eluate along with the monosaccharides. If, on the other hand, the column was prepared with the adsorbent containing a high proportion of charcoal, a very slow flow rate was obtained and the complete separation was difficult to carry out. Poor separation was also found with one batch of Darco G-60, but a number of subsequent batches have given no difficulty.

The desorption of a number of monosaccharides that might be expected in plant extracts was carried out and the results are shown in table I. Two different charcoal-Celite mixtures were prepared and tested for improved sugar recovery. The results of the desorption of glucose, fructose, and galactose from adsorbent columns prepared from the various charcoals are shown in table II. Bone charcoal gave much better recoveries than the other charcoals but, because of the difficulty in obtaining uniform batches for routine work, the Darco G-60 has been used in all subsequent work.

To check the completeness of the monosaccharide-disaccharide separation, two experiments were carried out with mixtures of C^{14}-labeled glucose

TABLE I

RECOVERY OF GLUCOSE, FRUCTOSE, GALACTOSE, AND ARABINOSE FROM A DARCO G-60–CELITE 535 COLUMN AFTER DESORPTION WITH 800 ml. OF WATER.

Sugar	Added	Recovered	Recovery
	mg.	*mg.*	%
Glucose	272	249	91
Fructose	259	259	100
Galactose	284	238	84
Arabinose	254	250	99

and sucrose. In the first case, 100 mg. of inactive glucose and 20 mg. of labeled sucrose (0.152 μc./mg.) were mixed, and then separated by the procedure described. The water and the 5% ethanol fractions were concentrated *in vacuo* to a small volume and placed on Whatman no. 1 filter paper for paper chromatography with a butanol-ethanol-water (40:10:50) solvent. Following the development of the chromatogram, the paper was dried and an autoradiograph was prepared. The developed autoradiograph revealed no sucrose spot in the monosaccharide fraction. Total and reducing sugar was determined chemically on the water and 5% ethanol fractions. The water fraction contained 92 mg. of reducing sugar and no non-reducing sugar. The 5% ethanol fraction contained 5 mg. of reducing sugar and 18 mg. of non-reducing sugar. A second mixture of 20 mg. of labeled glucose (0.64 μc./mg.) and 100 mg. of inactive sucrose was prepared and treated similarly. Autoradiographs of the two fractions indicated a small glucose contamination in the sucrose. The water fraction was found to contain 18 mg. of reducing sugar and no non-reducing sugar. The 5% ethanol fraction contained about 1 mg. of reducing and 94 mg. of non-reducing sugar.

Raffinose, the only trisaccharide commonly found in plant tissues, may be conveniently isolated from plant extracts by the method of Whistler and Durso. Using this technique we pooled a number of trisaccharide fractions from biosynthesis runs and isolated and crystallized the raffinose (**1**). At this writing a total of 600 mg. of C^{14}-labeled raffinose (0.021 μc./mg.) have been prepared.

TABLE II

RECOVERY OF GLUCOSE, FRUCTOSE, AND GALACTOSE FROM DARCO G-60, NORIT A AND BONE CHARCOAL COLUMNS AFTER DESORPTION WITH 800 ml. OF WATER.

Sugar	Darco G-60	Norit A	Bone charcoal
	%	%	%
Glucose	91	87	97
Fructose	100	90	100
Galactose	84	87	100

The leaves of *Canna indica* were exposed to 5 mc. of C^{14} as $C^{14}O_2$ in the photosynthesis apparatus described by NOGGLE and BOLOMEY (**4**), after which the sugars were extracted with 80% ethanol and then deproteinized and deionized (**4**). The extract, containing 300 mg. of sucrose and 230 mg. of reducing sugars was concentrated to 25 ml., and then separated on a Darco G-60-Celite 535 column. Paper chromatograms of the three separated fractions were prepared and then autoradiographed (fig. 1). The monosaccharide fraction was found to contain no sucrose. There was also a small spot indicating a compound with an R_f value lower than sucrose, but in an amount too small to identify. This compound did not appear to be raffinose but it moved on the paper to a region that was slightly above

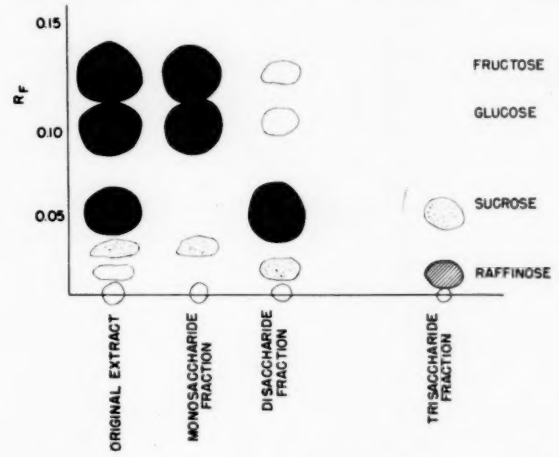

FIG. 1. Autoradiograph of papergram run in butanol-ethanol-water (40 : 10 : 50) containing fractions from sugar separation of *Canna indica* leaves.

raffinose. Phosphorylated sugars also are found in this region but the ion-exchange treatment should have removed them. The material did not interfere with the subsequent column separation of the glucose and fructose. The disaccharide fraction contained sucrose, a small amount of glucose, and some sugar that appeared to be raffinose. The trisaccharide fraction contained raffinose and a small amount of sucrose.

Another feature which enhances the value of the charcoal-Celite column when handling plant extracts is its clarifying power. Traces of color which frequently appear in the extract are partially removed in the deproteinization and deionization procedure. Such colored extracts, when passed through the charcoal-Celite columns, come out sparkling clear.

The problem of primary importance in the investigation was to find a method that would remove the disaccharides from plant extracts so that

the monosaccharides could be separated by column chromatography. The method of Whistler and Durso was found to be satisfactory for this purpose. In addition, the data of Whistler and Durso indicated that a disaccharide and a trisaccharide fraction might be separated from plant extracts. This was found to be true but there was some monosaccharide and trisaccharide contamination of the disaccharide fraction and a disaccharide contamination in the trisaccharide fraction. The amount of contamination was very small and under ordinary conditions would not interfere with the preparative separation of the sugar components from a plant extract. When handling labeled sugars, however, this contamination might give some trouble.

A second prerequisite of the method was that it be capable of handling at least one gram of sugar. The Whistler and Durso method also fulfilled this requirement.

The present method does not satisfactorily solve the problem of completely separating the sugar components from complex mixtures such as plant extracts. There are many important sugars such as the pentoses that can not be separated. The recent demonstration by KHYM and ZILL (**2**) that sugars could be separated by ion-exchange has opened up the possibility of making a complete separation of all the sugars present in plant extracts. The method has been adapted to the quantitative analysis of the sugars in plant extracts (**5**) and additional work has shown its application to preparative procedures.

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NOTES

Western Section.—Officers of the Western Section for the year 1951–52 are Herbert B. Currier, Chairman, M. D. Thomas, Vice-Chairman, and Harlan K. Pratt, Secretary-Treasurer. The Section will meet with other affiliates of the Pacific Division, A.A.A.S., at Oregon State College, Corvallis, June 17–20, 1952. The program will consist of four sessions of submitted papers, a field trip, and two symposia. Members of the Society who may be able to attend should correspond at once with the Secretary and Program Chairman of the Western Section to receive further notices or to submit papers for presentation. Address: Prof. Harlan K. Pratt, Division of Truck Crops, University of California, Davis, California.

John I. Lauritzen.—The American Society of Plant Physiologists records with sorrow the passing of one of its members, John Irwin Lauritzen, retired physiologist with the Bureau of Plant Industry. Dr. Lauritzen received his B.S. degree from Utah State College in 1914 and his Ph.D. in plant pathology from Cornell University in 1919. He served as a plant pathologist with the Bureau of Plant Industry from 1918 until 1930 and as Senior Physiologist from 1930 until his retirement. Dr. Lauritzen was a charter member of the American Society of Plant Physiologists and maintained his membership continuously from 1924 to 1951. He died in November 1951 at Riverside, California, at the age of 67.

Mineral Nutrition of Plants.—Emil Truog, Editor. University of Wisconsin Press, 811 State Street, Madison, Wisconsin. 469 pages, 1951. $6.00.

Mineral Nutrition of Plants comprises the papers presented during a symposium on mineral nutrition at the University of Wisconsin in September 1949. The symposium was arranged for the presentation of the latest theoretical and practical information by those actively engaged in research in mineral nutrition of plants. The topics included are: World Food Possibilities and Fertility Status of Our Soils by C. E. Kellogg and A. C. Orvedal; Soil as a Medium for Plant Growth by E. Truog; The Activities of Cations Held by Soil Colloids and the Chemical Environment of Plant Roots by C. E. Marshall; The Availability of Soil Anions by R. Overstreet and L. A. Dean; Contact Phenomena between Adsorbents and Their Significance in Plant Nutrition by H. Jenny; The Effect of Soil Physical Properties on Nutrient Availability by J. B. Page and G. B. Bodman; Role of Soil Microorganisms in Nutrient Availability by A. G. Norman; The Nature of the Process of Inorganic Solute Accumulation in Roots by T. C. Broyer; The Mechanism of Ion Absorption by H. Burström; The Translocation of Minerals in Plants by O. Biddulph; Control of Nitrogen Effects on McIntosh Apple Trees in New York by D. Boynton; Production of Vegetable

Crops for the Canning Industry by J. B. Hester; Growth and Function as Criteria in Determining the Essential Nature of Inorganic Nutrients by D. I. Arnon; Mineral Nutrition in Relation to the Ontogeny of Plants by W. F. Loehwing; Correlations between Protein-Carbohydrate Metabolism and Mineral Deficiencies in Plants by R. A. Steinberg; Light as a Modifying Influence on the Mineral Nutrition of Plants by R. B. Withrow; Soil Moisture and the Mineral Nutrition of Plants by C. H. Wadleigh and L. A. Richards; Environmental Influences on the Growth of Sugar Cane by H. F. Clements.

Bacterial Physiology.—Edited by C. H. Werkman and P. W. Wilson, Academic Press, Inc., 125 East 23rd Street, New York 10, New York. 707 pages, 1951. $8.50.

The contributors to this volume present the chemistry and physiology of the bacteria in general discussions of experimental data, modern views, and theories. The authoritative treatments of the fundamentals of bacterial physiology and its contributions in the broader fields of general biology will be of great value to teachers, advanced students and research workers in all of the plant sciences. The chapters are: Chemistry of the Bacterial Cell and the Structure of the Bacterial Cell by G. Knaysi; Inheritance, Variation, and Adaptation by J. Lederberg; Growth of Bacteria by I. C. Gunsalus; Physical Factors Affecting Growth and Death by P. Mitchell; Chemical Factors Affecting Growth and Death by O. Wyss; Bacterial Nutrition-Chemical Factors by E. E. Snell; Bacterial Enzymes and the Theory of Action by F. Schlenk; Anaerobic Dissimilation of Carbohydrates by C. H. Werkman and F. Schlenk; Bacterial Oxidations by E. S. G. Barron; Autotrophic Assimilation of Carbon Dioxide by J. W. Foster; Assimilation of Carbon Dioxide by Heterotrophic Bacteria by C. H. Werkman; Organic Nitrogen by E. F. Gale; Biological Nitrogen Fixation by P. W. Wilson; Mineral Metabolism by S. G. Knight; The Comparative Biochemistry of Molecular Hydrogen by H. Koffler and P. W. Wilson; Assimilation by Bacteria by C. E. Clifton; Degradation and Synthesis of Complex Carbohydrates by H. A. Barker and W. Z. Hassid; Significance of Autotrophy for Comparative Physiology by W. W. Umbreit; Luminous Bacteria by F. H. Johnson. Reference citations selected by the contributors are assembled in a bibliography for the entire volume. A subject index and a microörganism index are included in the book.

Natural and Synthetic High Polymers.—Kurt H. Meyer. Second Edition. Interscience Publishers, Inc., 250 Fifth Avenue, New York 1, New York. 891 pages, 1950. $15.00.

This publication is the second edition of the book which appeared in 1942. The rapid development of polymer chemistry in theoretical concepts, new groups of polymers and new applications has occasioned the rewriting of the text and the addition of over 200 pages of new material. The book is organized in the following sections: Physical and physico-chemical investigations of high polymers; Inorganic high polymers; High polymeric hydrocarbons and their derivatives; Polymeric ethers, esters, sulphides, etc.;

Cellulose and its derivatives; Animal and vegetable polysaccharides and lignin; Proteins; Properties of high polymers in solution; Elasticity, viscosity, and plasticity of high polymers; Films, foils and membranes; Molecular structure of animal and plant tissues. Author and subject indices are included.

Advances in Enzymology and Related Subjects of Biochemistry, Volume XII.—Edited by F. F. Nord. Interscience Publishers, Inc., 250 Fifth Avenue, New York 1, New York. 570 pages, 1951. $9.75.

The publishers of this series have deviated from their annual schedule by issuing an additional volume for 1951 in order to avoid a delay in publication of manuscripts on hand. The following articles are included in this volume: Oxidoreduction in Chloroplasts by Robert Hill; Mechanisms of Fixation of Carbon Dioxide by Heterotrophs and Autotrophs by Berton F. Utter and Harland G. Wood; Enzyme-Substrate Compounds by Britton Chance; The Specificity of Certain Peptidases by Emil L. Smith; The Enzymic Hydrolysis and Synthesis of Acetylcholine by David Nachmansohn and Irwin B. Wilson; The Present Status of Starch Chemistry by Kurt H. Meyer and G. C. Gibbons; Enzymes of Starch Degradation and Synthesis by Peter Bernfeld; Biological Methylation by Molds by F. Challenger; and Reaction of Borate with Substances of Biological Interest by Charles A. Zittle. Author and subject indices for this volume and a cumulative index of authors and subject for volumes one to twelve complete the book.

Advances in Agronomy, Volume III.—A. G. Norman, Editor. Academic Press, Inc., 125 East 23rd Street, New York 10, New York. 361 pages, 1951. $7.80.

The third volume in this series of publications includes the following articles: The Subsoil by E. Winters; The Management of Alfalfa Meadows by C. J. Willard; The Chemistry of Soil Potassium by R. F. Reitemeier; The Changing Pattern of Agriculture in England and Wales by J. A. McMillan; The Adaptability and Breeding of Suitable Grasses for the Southeastern States by G. W. Burton; Chemical Treatment of the Soil for Nematode Control by A. L. Taylor; Problems and Progress of Soil Conservation in the United States by H. J. Harper; Irrigation, Agriculture and Soil Research in the United States by R. O. Parks. Author and subject indices are included.

Industrial Oil and Fat Products.—Alton E. Bailey. Second Edition. Interscience Publishers, Inc., 250 Fifth Avenue, New York 1, New York. 967 pages, 1951. $15.00.

The revised and augmented edition of this work is again primarily a text on oil and fat technology with the greater part devoted to a description and discussion of the commercially important oil and fat products and the processes used in the manufacture of these products. In other sections the chemical and physical nature of fats and oils is briefly reviewed, and the various fatty raw materials are considered with respect to their composition,

characteristics, and availability. Author and subject indices complete the volume.

The Lipids, Volume I: Chemistry.—Harry J. Deuel, Jr. Interscience Publishers, Inc., 250 Fifth Avenue, New York 1, New York. 982 pages, 1951. $18.50.

The first volume of this two-volume treatise covers the chemistry of the lipids. The second volume will be concerned exclusively with the biochemical and nutritional implications of the substances. The information on the chemistry of the lipids in both plant and animal tissues is organized in the following units: General characteristics and classification of lipids; The chemistry of fatty acids and glycerol; The chemistry of neutral fats; waxes, higher alcohols including sterols, triterpenes, glyceryl, ethers, colored fats, and hydrocarbons; Chemistry of the phosphatides and cerebrosides; Carotenoids and related compounds; Distribution, properties, and chemistry of the vitamins A, provitamins D and vitamins D, the vitamin E group, and the vitamin K group. Comprehensive author and subject indices and an index to the generic names of the plant and animal sources of lipids are included in the volume.

Conference on the Use of Isotopes.—Kansas State College, Argonne National Laboratory, and the Isotopes Division of the United States Atomic Energy Commission will sponsor a conference on The Use of Isotopes in Plant and Animal Research, June 12 to June 14, 1952, at Kansas State College, Manhattan, Kansas. A detailed program will be available in March. Persons wishing to receive it, together with information on housing, should write Director R. I. Throckmorton, Kansas Agricultural Experiment Station, Manhattan, Kansas.

Principles of Plant Physiology.—James Bonner and Arthur W. Galston. W. H. Freeman and Company, San Francisco, California. 509 pages, 1952. $5.50.

This book meets a long standing need for an elementary textbook of plant physiology which gives an adequate treatment of the chemistry of plant processes, particularly those of metabolism. The basic principles of plant physiology are presented in a concise and coherent account designed especially for understanding by all students of the plant sciences. Such a presentation has necessitated the adoption of certain interpretations in controversial issues which the authors judge most helpful to the student. The numerous illustrations which are generally excellent and in some instances very dramatic contribute greatly to the value of the textbook. The illustrations of metabolic pathways in plants will be especially helpful to beginning students. The subject matter is arranged in twenty chapters under the three major headings of nutrition, metabolism, and growth and development. Original research papers are not cited in the text but each chapter contains a list of references for general reading which includes a review paper that provides the student with an entrance to the literature on the various topics. The book is provided with an index.

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

Organised in 1924 for the advancement of Plant Physiology as a pure and applied field of botanical science

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Any one actively interested in Plant Physiology is eligible for membership in the Society. Candidates for membership must be recommended by two members, and approved by executive committee, or by the vote of the Society at some annual or special meeting.

The payment of the annual dues to the Society entitles the members to receive the official journal, Plant Physiology. They will receive in addition the regular bulletins and announcements sent out periodically by the secretary. Members are invited to take a personal interest in the growth and development of the Society.

Manuscripts for Plant Physiology should be addressed to Prof. Walter F. Loehwing, Department of Botany, State University of Iowa, Iowa City, Iowa. Drawings and photographs should not exceed 8.5 × 11 inches in size, should be numbered correctly on the back, and separate typewritten legends included with the text of the manuscript. Tables and cuts should be used sparingly, and the text presented in concise form. Instructions to authors for preparation of manuscripts are given in the January number, Volume 27, 1952, of Plant Physiology, 221–222.

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